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(54) Title: DNA SEQUENCES, RECOMBINANT DNA MOLECULES AND PROCESSES FOR PRODUCING SOLUBLE T4 PROTEINS

(57) Abstract

This invention relates to DNA sequences, recombinant DNA molecules and processes for producing soluble T4 protein. More particularly, this invention relates to DNA sequences that are characterized in that they code on expression in an appropriate unicellular host for soluble forms of T4, the receptor on the surface of T4+ lymphocytes, or derivatives thereof. In accordance with this invention, the DNA sequences, recombinant DNA molecules and processes of this invention may be employed to produce soluble T4 essentially free of other proteins of human origin. This soluble protein may then advantageously be used in the immunotherapeutic and diagnostic compositions and methods of this invention. The soluble T4-based immunotherapeutic compositions and methods of this invention are useful in treating immunodeficient patients suffering from diseases caused by infective agents whose primary targets are T4+ lymphocytes. According to a preferred embodiment, this invention relates to soluble T4-based compositions and methods which are useful in preventing, treating or detecting acquired immune deficiency syndrome, AIDS related complex and HIV infection.

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(57) Abstract

This invention relates to DNA sequences, recombinant DNA molecules and processes for producing soluble T4 protein. More particularly, this invention relates to DNA sequences that are characterized in that they code on expression in an appropriate unicellular host for soluble forms of T4, the receptor on the surface of T4+ lymphocytes, or derivatives thereof. In accordance with this invention, the DNA sequences, recombinant DNA molecules and processes of this invention may be employed to produce soluble T4 essentially free of other proteins of human origin. This soluble protein may then advantageously be used in the immunotherapeutic and diagnostic compositions and methods of this invention. The soluble T4-based immunotherapeutic compositions and methods of this invention are useful in treating immunodeficient patients suffering from diseases caused by infective agents whose primary targets are T4+ lymphocytes. According to a preferred embodiment, this invention relates to soluble T4-based compositions and methods which are useful in preventing, treating or detecting acquired immune deficiency syndrome, AIDS related complex and HIV infection.

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DNA SEQUENCES, RECOMBINANT DNA MOLECULES AND PROCESSES FOR PRODUCING SOLUBLE T4 PROTEINS

TECHNICAL FIELD OF INVENTION

This invention relates to DNA sequences, recombinant DNA molecules and processes for producing soluble T4 proteins. More particularly, this invention relates to DNA sequences that are characterized in that they code on expression in an appropriate unicellular host for soluble forms of T4, the receptor on the surface of $T4^+$ lymphocytes, or derivatives thereof. In accordance with this invention, the DNA sequences, recombinant DNA molecules and processes of this invention may be employed to produce soluble T4 essentially free of other proteins of human origin. This soluble protein may then advantageously be used in the immunotherapeutic, prophylactic, and diagnostic compositions and methods of this invention.

The soluble T4 protein-based immunotherapeutic compositions and methods of this invention are useful in treating immunodeficient patients suffering from diseases caused by infective agents whose primary targets are $T4^+$ lymphocytes. According to a preferred embodiment, this invention relates to soluble T4 protein-based compositions and methods which are useful in preventing, treating or detecting

acquired immune deficiency syndrome, AIDS related complex and HIV infection.

BACKGROUND ART

The class of immune regulatory cells known as T cell lymphocytes can be divided into two broad functional classes, the first class comprising T helper or inducer cells -- which mediate T cell proliferation, lymphokine release and helper cell interactions for Ig release, and the second class comprising T cytotoxic or suppressor cells -- which participate in T cell-mediated killing and immune response suppression. In general, these two classes of lymphocytes are distinguished by expression of one of two surface glycoproteins: T4 (m.w. 55,000-62,000 daltons) which is expressed on T helper or inducer cells, probably as a monomeric protein, or T8 (m.w. 32,000 daltons) which is expressed on T cytotoxic or suppressor cells as a dimeric protein.

The primary structures of T4 and T8 have been deduced from their respective cDNA sequences [P. J. Maddon et al., "The Isolation and Nucleotide Sequence Of A cDNA Encoding The T Cell Surface Protein T4: A New Member Of The Immunoglobulin Gene Family", Cell, 42, pp. 93-104 (1985); D. R. Littman et al., "The Isolation And Sequence Of The Gene Encoding T8: A Molecule Defining Functional Classes Of T Lymphocytes", Cell, 40, pp. 237-46 (1985)]. Both predicted protein sequences define molecules with domains expected for surface antigens, including transmembrane and intracytoplasmic domains at the carboxyl end of the protein. In addition, both proteins contain an amino terminal region which shows striking homology to immunoglobulin and T cell receptor variable regions and which might function during target cell recognition [Maddon et al., supra].

In immunocompetent individuals, T4 lymphocytes interact with other specialized cell types of the immune system to confer immunity to or defense against infection [E. L. Reinherz and S. F. Schlossman, "The Differentiation Function Of Human T-Cells", Cell, 19, pp. 821-27 (1980)]. More specifically, T4 lymphocytes stimulate production of growth factors which are critical to a functional immune system. For example, they act to stimulate B cells, the descendants of hemopoietic stem cells, which promote the production of defensive antibodies. They also activate macrophages ("killer cells") to attack infected or otherwise abnormal host cells and they induce monocytes ("scavenger cells") to encompass and destroy invading microbes.

It has been found that the primary target of or receptor for certain infective agents is the T4 surface protein. These agents include, for example, viruses and retroviruses. When T4 lymphocytes are exposed to such agents, they are rendered nonfunctional. As a result, the host's complex immune defense system is destroyed and the host becomes susceptible to a wide range of opportunistic infections.

Such immunosuppression is seen in patients suffering from acquired immune deficiency syndrome ("AIDS"). AIDS is a disease characterized by severe or, typically, complete immunosuppression and attendant host susceptibility to a wide range of opportunistic infections and malignancies. In some cases, AIDS infection is accompanied by central nervous system disorders. Complete clinical manifestation of AIDS is usually preceded by AIDS related complex ("ARC"), a syndrome accompanied by symptoms such as persistent generalized lymphadenopathy, fever and weight loss. The human immunodeficiency virus ("HIV") retrovirus is thought to be

the etiological agent responsible for AIDS infection and its precursor, ARC [M. G. Sarngadharan et al., "Detection, Isolation And Continuous Production Of Cytopathic Retroviruses (HTLV-III) From Patients With AIDS And Pre-AIDS", Science, 224, pp. 497-508 (1984)].*

Between 85 and 100% of the AIDS/ARCS population test seropositive for HIV [G. N. Shaw et al., "Molecular Characterization Of Human T-Cell Leukemia (Lymphotropic) Virus Type III In The Acquired Immune Deficiency Syndrome", Science, 226, pp. 1165-70 (1984)]. The number of adults in the United States infected with HIV has been estimated to be between 1 and 2.5 million [D. Barnes, "Strategies For An AIDS Vaccine", Science, 233, pp. 1149-53 (1986); M. Rees, "The Sombre View Of AIDS", Nature, 326, pp. 343-45 (1987)]. These estimates include 64,900 individuals who do not belong to an identified group at risk for AIDS [S. L. Sivak and G. P. Wormser, "How Common Is HTLV-III Infection In The United States?", New Eng. J. Med., 313, p. 1352 (1985)]. The apparent annual rate of diagnosis for those infected with HIV virus is between 1 and 2% -- a rate which may increase significantly in future years.

The genome of retroviruses, such as HIV, contains three regions encoding structural proteins. The gag region encodes the core proteins of the virion. The pol region encodes the virion RNA-dependent DNA polymerase (reverse transcriptase). The

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* In this application, human immunodeficiency virus ("HIV"), the generic term adopted by the human retrovirus subcommittee of the International Committee On Taxonomy Of Viruses to refer to independent isolates from AIDS patients, including human T cell lymphotropic virus type III ("HTLV-III"), lymphadenopathy-associated virus ("LAV"), human immunodeficiency virus type 1 ("HIV-1") and AIDS-associated retrovirus ("ARV") will be used.

env region encodes the major glycoprotein found in the membrane envelope of the virus and in the cytoplasmic membrane of infected cells. The capacity of the virus to attach to target cell receptors and to cause fusion of cell membranes are two HIV properties controlled by the env gene. These properties are believed to play a fundamental role in the pathogenesis of the virus.

HIV env proteins arise from a precursor polypeptide that, in mature form, is cleaved into a large heavily glycosylated exterior membrane protein of about 481 amino acids -- gp120 -- and a smaller transmembrane protein of about 345 amino acids which may be glycosylated -- gp41 [L. Ratner et al., "Complete Nucleotide Sequence Of The AIDS Virus, HTLV-III", Nature, 313, pp. 277-84 (1985)].

The host range of the HIV virus is associated with cells which bear the surface glycoprotein T4. Such cells include T4 lymphocytes and brain cells [P. J. Maddon et al., "The T4 Gene Encodes The AIDS Virus Receptor And Is Expressed In The Immune System And The Brain", Cell, 47, pp. 333-48 (1986)]. Upon infection of a host by HIV virus, the T4 lymphocytes are rendered non-functional. The progression of AIDS/ARCS syndromes can be correlated with the depletion of $T4^+$ lymphocytes, which display the T4 surface glycoprotein. This T cell depletion, with ensuing immunological compromise, may be attributable to both recurrent cycles of infection and lytic growth from cell-mediated spread of the virus. In addition, clinical observations suggest that the HIV virus is directly responsible for the central nervous system disorders seen in many AIDS patients.

The tropism of the HIV virus for $T4^+$ cells is believed to be attributed to the role of the T4 cell surface glycoprotein as the membrane-anchored virus receptor. Because T4 behaves as the HIV virus

receptor, its extracellular sequence probably plays a direct role in binding HIV. More specifically, it is believed that HIV envelope selectively binds to the T4 epitope(s), using this interaction to initiate entry into the host cell [A. G. Dalgelish et al., "The CD4 (T4) Antigen Is An Essential Component Of The Receptor For The AIDS Retrovirus", Nature, 312, pp. 763-67 (1984); D. Klatzmann et al., "T-Lymphocyte T4 Molecule Behaves As The Receptor For Human Retrovirus LAV", Nature, 312, pp. 767-68 (1984)]. Accordingly, cellular expression of T4 is believed to be sufficient for HIV binding, with the T4 protein serving as a receptor for the HIV virus.

The T4 tropism of the HIV virus has been demonstrated in vitro. When HIV virus isolated from AIDS patients is cultured together with T helper lymphocytes preselected for surface T4, the lymphocytes are efficiently infected, display cytopathic effects, including multinuclear syncytia formation and are killed by lytic growth [D. Klatzmann et al., "Selective Tropism Of Lymphadenopathy Associated Virus (LAV) For Helper-Inducer T Lymphocytes", Science, 225, pp. 59-63 (1984); F. Wong-Staal and R. C. Gallo, "Human T-Lymphotropic Retroviruses", Nature, 317, pp. 395-403 (1985)]. It has been demonstrated that a cloned cDNA version of human T4, when expressed on the surface of transfected cells from non-T cell lineages, including murine and fibroblastoid cells, endows those cells with the ability to bind HIV [P. J. Maddon et al., "The T4 Gene Encodes The AIDS Virus Receptor And Is Expressed In The Immune System And The Brain", Cell, 47, pp. 333-48 (1986)].

During the course of HIV infection, the host mounts both a humoral and a cellular immune response to the virus. These responses include the appearance of antibodies which bind to a number of viral products and which exhibit neutralizing effect

or antibody dependent cellular cytotoxic functions [M. Guroff-Robert et al., "HTLV-III-Neutralizing Antibodies In Patients With AIDS And AIDS-Related Complex", Nature, 316, pp. 72-74 (1985); D. D. F. Barin et al., "Virus Envelope Protein Of HTLV-III Represents Major Target Antigen For Antibodies In AIDS Patients", Science, 228, pp. 1094-96 (1985); A. H. Rook et al., "Sera From HTLV-III/LAV Antibody Positive Individuals Mediate Antibody Dependent Cellular Cytotoxicity Against HTLV-III/LAV Infected T Cells", J. Immunol., 138, pp. 1064-68 (1987)]. Epitopes of the HIV envelope have been identified as important determinants in eliciting a neutralizing antibody response. And, determinants in antibody dependent cellular cytotoxicity ("ADCC") activity include HIV env and, possibly, gag epitopes.

In the absence to date of effective treatments for AIDS, many efforts have centered on prevention of the disease. Such preventative measures include HIV antibody screening for all blood, organ and semen donors and education of AIDS high-risk groups regarding transmission of the disease.

Experimental or early-stage clinical treatment of AIDS and ARCS conditions have included the administration of antiviral drugs, such as HPA-23, phosphonoformate, suramin, ribavirin, azidothymidine ("AZT") and dideoxycytidine, which apparently interfere with replication of the virus through reverse transcriptase inhibition. Although each of these drugs exhibits activity against HIV in vitro, only AZT has demonstrated potential benefits in clinical trials. AZT administration in effective amounts, however, has been accompanied by undesirable and debilitating side effects, such as bone marrow depression. It is likely, therefore, that hematologic toxicity will be a major rate limiting factor in the long term use of AZT.

Other proposed methods for treating AIDS have focused on the development of agents having activity against steps in the viral replicative cycle other than reverse transcription. Such methods include the administration of interferons or the application of hybridoma technology. Most of these treatment strategies are expected to require the co-administration of immunomodulators, such as interleukin-2.

To date, the need exists for the development of effective immunotherapeutic agents and methods for the treatment of AIDS, ARCS, HIV infection and other immunodeficiencies caused by T lymphocyte depletion or abnormalities.

DISCLOSURE OF THE INVENTION

The present invention solves the problems referred to above by providing, in large amounts, soluble T4 and soluble derivatives thereof that act as receptors for infective agents whose primary target is the T4 surface protein of $T4^+$ lymphocytes. Advantageously, this invention also provides soluble T4 essentially free of other proteins of human origin and in a form that is not contaminated by viruses, such as HIV or hepatitis B virus.

As will be appreciated from the disclosure to follow, the DNA sequences and recombinant DNA molecules of this invention are capable of directing, in an appropriate host, the production of soluble T4 or derivatives thereof. The polypeptides of this invention are useful, either as produced in the host or after further derivatization or modification, in a variety of immunotherapeutic compositions and methods for treating immunodeficient patients suffering from diseases caused by infective agents whose primary targets are $T4^+$ lymphocytes. According to various embodiments of this invention, such compo-

sitions and methods relate to a soluble receptor for HIV, soluble T4 proteins and polypeptides and antibodies thereto. The soluble T4 proteins and polypeptides of this invention include monovalent, as well as polyvalent forms.

The compositions and methods of this invention, which are based upon soluble T4 proteins, polypeptides or peptides and antibodies thereto, are particularly useful for the prevention, treatment or detection of the HIV-related infections AIDS and ARC. More specifically, the soluble T4-based compositions and methods of this invention employ soluble T4-like polypeptides -- polypeptides which advantageously interfere with the T4/HIV interaction by blocking or competitive binding mechanisms which inhibit HIV infection of cells expressing the T4 surface protein. These soluble T4-like polypeptides inhibit adhesion between $T4^+$ lymphocytes and infective agents which target $T4^+$ lymphocytes and inhibit interaction between $T4^+$ lymphocytes and antigen presenting cells and targets of $T4^+$ lymphocytes mediated killing. By acting as soluble virus receptors, the compositions of this invention may be used as antiviral therapeutics to inhibit HIV binding to $T4^+$ cells and virally induced syncytium formation at the level of receptor binding.

This invention accomplishes these goals by providing DNA sequences coding on expression in an appropriate unicellular host for soluble T4 proteins* and soluble derivatives thereof.

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* As used in this application, "soluble T4 protein", "soluble T4" and "soluble T4-like polypeptides" include all proteins, polypeptides and peptides which are natural or recombinant soluble T4 proteins, or soluble derivatives thereof, and which are characterized by the immunotherapeutic (anti-retroviral)

(footnote continued on following page)

This invention also provides recombinant DNA molecules containing those DNA sequences and unicellular hosts transformed with them. Those hosts permit the production of large quantities of the novel soluble T4 proteins, polypeptides, peptides and derivatives of this invention for use in a wide variety of therapeutic, prophylactic and diagnostic compositions and methods.

The DNA sequences of this invention are selected from the group consisting of:

(a) the DNA inserts of p199-7, pBG377, pBG380, pBG381, p203-5, pBG391, pBG392, pBG393, pBG394, pBG395, pBG396, pBG397, p211-11, p214-10 and p215-7;

(b) DNA sequences which hybridize to one or more of the foregoing DNA inserts and which code on expression for a soluble T4-like polypeptide; and

(c) DNA sequences which code on expression for a soluble T4-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

According to an alternate embodiment, this invention also relates to a DNA sequence comprising the DNA insert of p170-2, said sequence coding on expression for a T4-like polypeptide. And, this invention also relates to recombinant DNA molecules and processes for producing T4 protein using that DNA sequence.

(footnote continued from preceding page)

or immunogenic activity of soluble T4 protein. They include soluble T4-like compounds from a variety of sources, such as soluble T4 protein derived from natural sources, recombinant soluble T4 protein and synthetic or semi-synthetic soluble T4 protein.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is an autoradiograph depicting the purification of T4 protein from U937 cells by immunoaffinity chromatography.

Figure 2 depicts autoradiograph and Western blot data demonstrating that immunoaffinity-purified, solubilized native T4 protein binds to HIV envelope protein.

Figure 3 depicts the nucleotide sequence and the derived amino acid sequence of T4 cDNA obtained from PBL clone λ203-4. In this figure, the amino acids are represented by single letter codes as follows:

Phe: F	Leu: L	Ile: I	Met: M
Val: V	Ser: S	Pro: P	Thr: T
Ala: A	Tyr: Y	His: H	Gln: Q
Asn: N	Lys: K	Asp: D	Glu: E
Cys: C	Trp: W	Arg: R	Gly: G

* = position at which a stop codon is present.

In Figure 3, the T4 protein translation start (AA_{-23}) is located at the methionine at nucleotides 201-203 and the mature N-terminus is located at the lysine (AA_3) at nucleotides 276-278.

Figure 4 is a schematic outline of the construction of cDNA clones pBG312.T4 (also called p171-1) and p170-2.

Figure 5 is a schematic outline of the construction of plasmid pEC100.

Figure 6 depicts amino acid comparisons at positions 3, 64 and 231 of various T4 cDNA clones.

Figures 7A and 7B depict the protein domain structure of purified, solubilized T4 protein and recombinant soluble T4 mutants.

Figures 8A-8D are schematic outlines of constructions of various intermediate plasmids and other plasmids used to express recombinant soluble T4 ("rsT4") of this invention.

Figure 9A is a schematic outline of the construction of plasmid p199-7.

Figures 9B and 9C are schematic outlines of the construction of plasmid p203-5.

Figure 10 depicts the synthetic oligonucleotide linkers employed in various constructions according to this invention.

Figure 11 depicts the nucleotide sequence of the entire plasmid defined by p199-7 ($P_L$mutet.rsT4) and its rsT4.2 insert and the amino acid sequence deduced from the rsT4 sequence. This includes the ClaI-ClaI cassette which defines the Met perfect rsT4.2 coding sequence.

Figure 12 depicts a protein blot analysis of an induction of rsT4.2 expression from SG936/p199-7.

Figure 13 is a schematic outline of the construction of plasmid pBG368.

Figures 14A-14C are schematic outlines of constructions of various plasmids of this invention.

Figure 15 depicts the nucleotide sequence of plasmid pBG391.

Figure 16 depicts the nucleotide sequence of plasmid pBG392. In this figure, the T4 protein translation start (AA_{-23}) is located at the methionine at nucleotides 1207-1209 and the mature N-terminus is located at the lysine (AA_3) at nucleotide 1281-84.

Figure 17 is a schematic outline of constructions of various plasmids of this invention.

Figure 18 depicts the synthetic oligonucleotide linkers employed in various constructions according to this invention.

Figure 19 depicts the nucleotide sequence of plasmid pBG394.

Figure 20 depicts the nucleotide sequence of plasmid pBG396.

Figure 21 depicts the nucleotide sequence of plasmid pBG393.

Figure 22 depicts the nucleotide sequence of plasmid pBG395.

Figure 23 is a Coomassie stained gel of rsT4.2 purified from the conditioned medium of the pBG380 transfected CHO cell line BG380G of plasmid p196-10.

Figure 24 is a schematic outline of the construction of plasmid p196-10.

Figure 25 is a schematic outline of the construction of plasmid pBG394.

Figure 26 is a schematic outline of the construction of plasmid p211-11.

Figure 27 is a schematic outline of the construction of plasmid p215-7.

Figure 28 is a schematic outline of the construction of plasmid p218-8.

Figure 29A is a Coomassie stained gel of rsT4.113.1 purified from the conditioned medium of pBG211-11 transfected E.coli.

Figure 29B is an autoradiograph depicting a Western blot analysis of rsT4.113.1 expressed in E.coli.

Figure 30, panels (a)-(c) depict the purification of rsT4.113.1 from E.coli transformants.

Figure 31, panels (a)-(c) depict the refolding of purified rsT4.113.1.

Figure 32 is an autoradiograph depicting the immunoprecipitation of ^{35}S-metabolically labelled CHO cell lines producing recombinant soluble T4.

Figure 33 depicts an immunoblot analysis of COS 7 cell lines producing recombinant soluble T4.

Figure 34 depicts in graphic form the results of a competition assay between rsT4.113.1 and rsT4.3 for binding to OKT4A or OKT4.

Figures 35-37 depict in graphic form the results of competition assays between rsT4.111 and rsT4.3 for binding to, respectively, OKT4A, Leu-3A and OKT4.

Figure 38 depicts in graphic form an ELISA assay for rsT4.113.1 from E.coli transformants.

Figure 39 depicts in graphic form the results of a p24 radioimmunoassay using recombinant soluble T4 according to this invention.

Figures 40 and 41 depict the results of syncytia inhibition assays using recombinant soluble T4 proteins according to this invention.

Figure 42 is a schematic outline of the construction of plasmid pBiv.1.

Figure 43 depicts the bivalent recombinant soluble T4 protein produced by pBiv.1.

DETAILED DESCRIPTION OF THE INVENTION

We isolated the DNA sequences of this invention from two libraries: a λgt cDNA library derived the T cell tumor line REX and a λgt10 cDNA library derived from peripheral blood lymphocytes. However, we could also have employed libraries prepared from other cells that express T4. These include, for example, H9 and U937. We also used a human genomic bank to isolate various fragments of the T4 gene.

For screening these libraries, we used a series of chemically synthesized anti-sense oligonucleotide DNA probes based upon the T4 protein sequence set forth in Maddon et al. (1985), supra.

For screening, we hybridized our oligonucleotide probes to our cDNA libraries utilizing a plaque hybridization screening assay. We selected clones hybridizing to several of our probes. And, after isolating and subcloning the cDNA inserts of the selected clones into plasmids, we determined

their nucleotide sequences and compared the amino acid sequences deduced from those nucleotide sequences to the amino acid sequences referred to in Maddon et al. (1985), supra. As a result of these comparisons, we determined that all of our selected clones were characterized by cDNA inserts coding for amino acid sequences of human T4.

We have depicted in Figure 3 the nucleotide sequence of full-length T4 cDNA obtained from deposited clone p170-2 and the amino acid sequence deduced therefrom. That cDNA sequence was subsequently subjected to in vitro site-directed mutagenesis and restriction fragment substitution so that its cDNA sequence was identical to that of Maddon et al.

After modifying our T4 cDNA sequence to be identical to that of Maddon et al., we truncated samples of it in various positions to remove the coding regions for the transmembrane and intracytoplasmic domains. The remaining cDNA sequences encoded a soluble T4 which retained the extracellular region believed to be responsible for HIV binding.

We then constructed various clones characterized by such cDNA inserts coding for human soluble T4. Those cDNA sequences may be used in a variety of ways in accordance with this invention. More particularly, those sequences or portions of them, or synthetic or semi-synthetic copies of them, may be used as DNA probes to screen other human or animal cDNA or genomic libraries to select by hybridization other DNA sequences that are related to soluble T4. Typically, conventional hybridization conditions, e.g., about 20° to 27°C below Tm, are employed in such selections. However, less stringent conditions may be necessary when the library is being screened with a probe from a different species than that from

which the library is derived, e.g., the screening of a mouse library with a human probe.

Such cDNA inserts, portions of them, or synthetic or semi-synthetic copies of them, may also be used as starting materials to prepare various mutations. Such mutations may be either degenerate, i.e., the mutation does not change the amino acid sequence encoded by the mutated codon, or non-degenerate, i.e., the mutation changes the amino acid sequence encoded by the mutated codon. Both types of mutations may be advantageous in producing or using soluble T4's according to this invention. For example, these mutations may permit higher levels of production or easier purification of soluble T4 or higher T4 activity.

For all of these reasons, the DNA sequences of this invention are selected from the group consisting of:

(a) the DNA inserts of p199-7, pBG377, pBG380, pBG381, p203-5, pBG391, pBG392, pBG393, pBG394, pBG395, pBG396, pBG397, p211-11, p214-10 and p215-7;

(b) DNA sequences which hybridize to one or more of the foregoing DNA inserts and which code on expression for a soluble T4-like polypeptide; and

(c) DNA sequences which code on expression for a soluble T4-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

Preferably, the DNA sequences of this invention code for a polypeptide selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{362} of Figure 3, a polypeptide of the formula AA_{1-362} of Figure 3, a polypeptide of the formula Met-AA_{1-362} of Figure 3, a polypeptide of the formula AA_{1-374} of Figure 3, a polypeptide of the formula Met-AA_{1-374} of Figure 3, a polypeptide of the formula AA_{1-377} of Figure 3, a polypeptide of the formula

Met-AA_{1-377} of Figure 3, a polypeptide of the formula AA_{-23}-AA_{374} of Figure 3, a polypeptide of the formula AA_{-23}-AA_{377} of Figure 3, or portions thereof.

DNA sequences according to this invention also preferably code for a polypeptide selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{182} of Figure 16, a polypeptide of the formula AA_1-AA_{182} of Figure 16, a polypeptide of the formula Met-AA_{1-182} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_1-AA_{182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula Met-AA_{1-182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_{-23}-AA_{113} of Figure 16, a polypeptide of the formula AA_1-AA_{113} of Figure 16, a polypeptide of the formula Met-AA_{1-113} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{111} of Figure 16, a polypeptide of the formula AA_1-AA_{111} of Figure 16, a polypeptide of the formula Met-AA_{1-111} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{131} of Figure 16, a polypeptide of the formula AA_1-AA_{131} of Figure 16, a polypeptide of the formula Met-AA_{1-131} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{145} of Figure 16, a polypeptide of the formula AA_1-AA_{145} of Figure 16, a polypeptide of the formula Met-AA_{1-145} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{166} of Figure 16, a polypeptide of the formula AA_1-AA_{166} of Figure 16, a polypeptide of the formula Met-AA_{1-166} of Figure 16, or portions thereof.

Additionally, DNA sequences of this invention code for a polypeptide selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{362} of mature T4 protein, a polypeptide of the formula

AA_{1-362} of mature T4 protein, a polypeptide of the formula Met-AA_{1-362} of mature T4 protein, a polypeptide of the formula AA_{1-374} of mature T4 protein, a polypeptide of the formula Met-AA_{1-374} of mature T4 protein, a polypeptide of the formula AA_{1-377} of mature T4 protein, a polypeptide of the formula Met-AA_{1-377} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{374} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{377} of mature T4 protein, or portions thereof.

DNA sequences according to this invention also code for a polypeptide selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{182} of mature T4 protein, a polypeptide of the formula AA_{1}-AA_{182} of mature T4 protein, a polypeptide of the formula Met-AA_{1-182} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_{1}-AA_{182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula Met-AA_{1-182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_{-23}-AA_{113} of mature T4 protein, a polypeptide of the formula AA_{1}-AA_{113} of mature T4 protein, a polypeptide of the formula Met-AA_{1-113} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{111} of mature T4 protein, a polypeptide of the formula AA_{1}-AA_{111} of mature T4 protein, a polypeptide of the formula Met-AA_{1-111} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{131} of mature T4 protein, a polypeptide of the formula AA_{1}-AA_{131} of mature T4 protein, a polypeptide of the formula Met-AA_{1-131} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{145} of mature T4 protein, a polypeptide of

the formula AA_1-AA_{145} of mature T4 protein, a polypeptide of the formula Met-AA_{1-145} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{166} of mature T4 protein, a polypeptide of the formula AA_1-AA_{166} of mature T4 protein, a polypeptide of the formula Met-AA_{1-166} of mature T4 protein, or portions thereof.

The amino terminal amino acid of mature T4 protein isolated from T cells begins at lysine, the third amino acid of the sequence depicted in Figure 16. Accordingly, soluble T4 proteins also include polypeptides of the formula AA_3-AA_{377} of Figure 16, or portions thereof. Such polypeptides include polypeptides selected from the group consisting of a polypeptide of the formula AA_3 to AA_{362} of Figure 16, a polypeptide of the formula AA_3 to AA_{374} of Figure 16, a polypeptide of the formula AA_3-AA_{182} of Figure 16, a polypeptide of the formula AA_3-AA_{113} of Figure 16, a polypeptide of the formula AA_3-AA_{131} of Figure 16, a polypeptide of the formula AA_3-AA_{145} of Figure 16, a polypeptide of the formula AA_3-AA_{166} of Figure 16, and a polypeptide of the formula AA_3-AA_{111} of Figure 16. Soluble T4 proteins also include the above-recited polypeptides preceded by an N-terminal methionine group.

Soluble T4 protein constructs according to this invention may also be produced by truncating the full length T4 protein sequence at various positions to remove the coding regions for the transmembrane and intracytoplasmic domains, while retaining the extracellular region believed to be responsible for HIV binding. More particularly, soluble T4 polypeptides may be produced by conventional techniques of oligonucleotide directed mutagenesis; restriction digestion, followed by insertion of linkers; or chewing back full length T4 protein with enzymes.

Alternatively, soluble T4 polypeptides may be chemically synthesized by conventional peptide synthesis techniques, such as solid phase synthesis [R. B. Merrifield, "Solid Phase Peptide Synthesis. I. The Synthesis Of A Tetrapeptide", J. Am. Chem. Soc., 83, pp. 2149-54 (1963)].

The DNA sequences of this invention code for soluble proteins and derivatives that are believed to bind to Major Histocompatibility Complex antigens and envelope glycoprotein of certain retroviruses, such as HIV. Preferably, they also inhibit syncytium formation, believed to be the mode of intracellular HIV virus spread. And, they may inhibit interaction between $T4^+$ lymphocytes and antigen-presenting cells and targets of $T4^+$ cell mediated killing. Most preferably, they also inhibit adhesion between $T4^+$ lymphocytes and infective agents, such as the HIV virus, whose primary targets are $T4^+$ lymphocytes.

The DNA sequences of this invention are also useful for producing soluble T4 or its derivatives coded for on expression by them in unicellular hosts transformed with those DNA sequences. As well known in the art, for expression of the DNA sequences of this invention, the DNA sequence should be operatively linked to an expression control sequence in an appropriate expression vector and employed in that expression vector to transform an appropriate unicellular host.

Such operative linking of a DNA sequence of this invention to an expression control sequence, of course, includes the provision of a translation start signal in the correct reading frame upstream of the DNA sequence. If the particular DNA sequence of this invention being expressed does not begin with a methionine, the start signal will result in an additional amino acid -- methionine -- being located at the N-terminus of the product. While

such methionyl-containing product may be employed directly in the compositions and methods of this invention, it is usually more desirable to remove the methionine before use. Methods are available in the art to remove such N-terminal methionines from polypeptides expressed with them. For example, certain hosts and fermentation conditions permit removal of substantially all of the N-terminal methionine in vivo. Other hosts require in vitro removal of the N-terminal methionine. However, such in vivo and in vitro methods are well known in the art.

A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from E.coli including col E1, pCR1, pBR322, pMB9 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs, e.g., the numerous derivatives of phage λ, e.g., NM989, and other DNA phages, e.g., M13 and filamenteous single stranded DNA phages, yeast plasmids, such as the 2μ plasmid or derivatives thereof, and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences. For animal cell expression, we prefer to use plasmid pBG368, a derivative of pBG312 [R. Cate et al., "Isolation Of The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986)] which contains the major late promoter of adenovirus 2.

In addition, any of a wide variety of expression control sequences -- sequences that con-

trol the expression of a DNA sequence when operatively linked to it -- may be used in these vectors to express the DNA sequence of this invention. Such useful expression control sequences, include, for example, the early and late promoters of SV40 or the adenovirus, the lac system, the trp system, the TAC or TRC system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, e.g., Pho5, the promoters of the yeast α-mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. For animal cell expression, we prefer to use an expression control sequence derived from the major late promoter of adenovirus 2.

A wide variety of unicellular host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of E.coli, Pseudomonas, Bacillus, Streptomyces, fungi, such as yeasts, and animal cells, such as CHO and mouse cells, African green monkey cells, such as COS 1, COS 7, BSC 1, BSC 40, and BMT 10, insect cells, and human cells and plant cells in tissue culture. For animal cell expression, we prefer CHO cells and COS 7 cells.

It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences of this invention. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences, and hosts without undue experimentation and without

departing from the scope of this invention. For example, in selecting a vector, the host must be considered because the vector must replicate in it. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the system, its controllability, and its compatibility with the particular DNA sequence of this invention, particularly as regards potential secondary structures. Unicellular hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for on expression by the DNA sequences of this invention to them, their secretion characteristics, their ability to fold proteins correctly, their fermentation requirements, and the ease of purification of the products coded on expression by the DNA sequences of this invention.

Within these parameters, one of skill in the art may select various vector/expression control system/host combinations that will express the DNA sequences of this invention on fermentation or in large scale animal culture, e.g., CHO cells or COS 7 cells.

The polypeptides produced on expression of the DNA sequences of this invention may be isolated from the fermentation or animal cell cultures and purified using any of a variety of conventional methods. One of skill in the art may select the most appropriate isolation and purification techniques without departing from the scope of this invention.

The polypeptides produced on expression of the DNA sequences of this invention are essentially

free of other proteins of human origin. Thus, they are different than T4 protein purified from human lymphocytes.

The polypeptides of this invention are useful in immunotherapeutic compositions and methods. For example, the polypeptides of this invention are active in inhibiting infection by agents whose primary targets are $T4^+$ lymphocytes by interfering with their interaction with those target lymphocytes. More preferably, the polypeptides of this invention may be employed to saturate the T4 receptor sites of T4-targeted infective agents. Thus, they exert anti-viral activity by competitive binding with cell surface T4 receptor sites. This effect is plainly of great utility in diseases, such as AIDS, ARC and HIV infection. Accordingly, the polypeptides and methods of this invention may be used to treat humans having AIDS, ARC, HIV infection or antibodies to HIV. In addition, these polypeptides and methods may be used for treating AIDS-like diseases caused by retroviruses, such as simian immunodeficiency viruses, in mammals, including humans.

According to one embodiment of this invention, antibodies to soluble T4 proteins and polypeptides may be used in the treatment, prevention, or diagnosis of AIDS, ARC and HIV infection.

The polypeptides of this invention may also be used in combination with other therapeutics used in the treatment of AIDS, ARC and HIV infection. For example, soluble T4 polypeptides may be used in combination with anti-retroviral agents that block reverse transcriptase, such as AZT, HPA-23, phosphonoformate, suramin, ribavirin and dideoxycitidine. Additionally, these polypeptides may be used with anti-viral agents such as interferons, including alpha interferon, beta interferon and gamma interferon, or glucosidase inhibitors, such as

castanospermine. Such combination therapies advantageously utilize lower dosages of those agents, thus avoiding possible toxicity.

And, the polypeptides of this invention may be used in plasmapheresis techniques or in blood bags for selective removal of viral contaminants from blood. According to this embodiment of the invention, soluble T4 polypeptides may be coupled to a solid support, comprising, for example, plastic or glass beads, or a filter, which is incorporated into a plasmapheresis unit.

Additionally, the compositions of this invention may be employed as immunosuppressants useful in preventing or treating graft-vs-host disease, autoimmune diseases and allograft rejection.

The compositions of this invention typically comprise an immunotherapeutic effective amount of a polypeptide of this invention and a pharmaceutically acceptable carrier. Therapeutic methods of this invention comprise the step of treating patients in a pharmaceutically acceptable manner with those compositions.

The compositions of this invention for use in these therapies may be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspensions, liposomes, suppositories, injectable and infusable solutions. The preferred form depends on the intended mode of administration and therapeutic application. The compositions also preferably include conventional pharmaceutically acceptable carriers and adjuvants which are known to those of skill in the art.

Generally, the pharmaceutical compositions of the present invention may be formulated and administered using methods and compositions similar to those used for other pharmaceutically important poly-

peptides (e.g., alpha-interferon). Thus, the polypeptides may be stored in lyophilized form, reconstituted with sterile water just prior to administration, and administered by the usual routes of administration such as parenteral, subcutaneous, intravenous, intramuscular or intralesional routes. An effective dosage may be in the range of from 0.5 to 5.0 mg/kg body weight/day, it being recognized that lower and higher doses may also be useful.

This invention also relates to soluble receptors and their use in diagnosing or treating viral agents which target or bind to those receptors. Such soluble receptors may be used as decoys to absorb viral agents and to halt the spread of viral infection. Alternatively, virus-killing agents may be attached to the soluble protein receptors, providing a direct mode of delivery of those agents to the virus.

More particularly, the polypeptides of this invention are useful in diagnostic compositions and methods to detect or monitor the course of HIV infection. Advantageously, these polypeptides are useful in diagnosing variants of the HIV virus, regardless of origin of the infecting HIV agent.

For example, soluble T4 proteins and polypeptides according to this invention, which have a high affinity for HIV, may be advantageously used to increase the sensitivity of HIV assay systems now based upon monoclonal or polyclonal antibodies. More specifically, soluble T4 proteins and polypeptides may be used to pretreat test plasma to concentrate any HIV present, even in small amounts, so that it is more easily recognized by the antibody. And soluble T4 proteins and polypeptides may be used to purify the HIV envelope protein gp120.

Alternatively, the soluble T4 proteins and polypeptides of this invention may be used to replace

anti-HIV antibodies now used in various assays. These soluble T4 proteins and polypeptides are be preferable to anti-HIV antibodies for two reasons. First, soluble T4, exhibits an affinity for HIV of approximately 10^{-9}, a level which exceeds the 10^{-7} to 10^{-8} values of anti-HIV antibodies. And, while anti-HIV antibodies are more likely to be specific for different HIV isolates, strain variations would not affect a soluble T4 protein-based assay, since all HIV isolates must be capable of interacting with the T4 receptor as a prerequisite to infectivity.

For example, a soluble T4 protein or polypeptide may be linked to an indicator, such as an enzyme, and used in an ELISA assay. Here, soluble T4 advantageously acts as a measure of both HIV in a test sample and any free HIV envelope gp120 protein.

And, polyvalent forms of soluble T4 proteins or polypeptides may be produced, for example, by chemical coupling or genetic fusion techniques, thus increasing even further the avidity of soluble T4 for HIV.

In order that this invention may be better understood, the following examples are set forth. These examples are for purposes of illustration only, and are not to be construed as limiting the scope of the invention in any manner.

EXAMPLES

Purification Of Native Solubilized T4

We purified native T4 from the $T4^+$-promonocytic cell line U937 derived from a histocytic lymphoma to approximately 50% purity using immunoaffinity chromatography as follows.

We grew U937 cells [a gift from Dr. Scott Hammer, New England Deaconess Hospital] to 10^6 cells/ml in RPMI 1640, 10% FCS, harvested and washed them in 1X PBS. We then lysed the cell pellet

in 20 mM Tris-HCl (pH 7.7), 0.5% NP-40 (a non-ionic detergent), 0.2% NaDOC, 0.2 mM EGTA, 0.2 mM PMSF and 5 µg/ml BPTI at 4 x 10^7 cells/ml. Because this purification was carried out in the presence of a non-ionic detergent, T4, which is normally membrane-bound via its hydrophobic transmembrane domain, was isolated as a solubilized protein. We spun the lysate in a GS 3 rotor for 10 min at 10,000 rpm and stored the supernatant at -70°C.

Subsequently, we preabsorbed the clarified cell extract with mouse IgG-Sepharose, followed by protein A Sepharose and then passed the flowthrough through an immunoaffinity column comprising immobilized 19Thy anti-T4 monoclonal antibody on Affigel-10 [a gift from Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, Massachusetts]. We washed the column extensively and eluted the bound material with 50 mM glycine-HCl (pH 2.5), 0.15 M NaCl, 0.5% NP-40, 5 µg/ml BPTI and 0.2 mM EGTA.

We then separated 10 µl aliquots of each elution fraction on a 10% SDS-PAGE under reducing conditions, with the bands being visualized by silver staining. As shown in Figure 1, a major silver-stained band of 55 Kd was visible. We then carried out two assays on the 55 Kd protein and sequenced the amino terminus of the protein to confirm its identity as native solubilized T4.

Sequencing Of Native Solubilized T4

We determined the N-terminal amino acid sequence of our solubilized native T4 which we isolated from a detergent extract of U937 cells by immunoaffinity chromatography as described above.

Techniques for determining the amino acid sequences of various proteins and peptides derived from them are well known in the art. We chose automated Edman degradation to determine the amino

terminus of our solubilized native T4. More specifically, we gel purified and electroeluted approximately 5 µg of the solubilized native T4 and then subjected it to automated Edman degradation using a gas phase sequencer (Applied Biosystems 470A). We then identified the PTH-amino acids produced at each cycle of the Edman chemistry by high pressure liquid chromatography, on-line with the sequencer, in a PTH-amino acid analyzer (Applied Biosystems 120A). Direct analysis of the protein provided amino terminal sequence information which, when compared to the amino acid sequence deduced from the cDNA sequence of human T4 [Maddon et al. (1985), supra], identified the purified protein as human T4.

Radioimmunoassay Of Native Solubilized T4

To determine that our purification process enriched for T4, we assayed fractions from the immunoaffinity elution step in a T4-specific sandwich radioimmunoassay, based upon the ELISA assay of P. E. Rao et al., in Cellular Immunology, 80, pp. 310-19 (1983). We coated each well of a Removawell strip (Dynatech Labs, Alexandria, Virginia) with 50 µl of 10 µl/ml OKT4 antibody (ATCC #CRL 8002) or MOPC195 (a background binding control) in 0.05 M sodium bicarbonate buffer (pH 9.4) at 4°C overnight. We washed the wells and then filled them with 1% FCS in PBS to saturate the protein binding capacity of the plastic. After removing the 1% FCS solution, we added test samples, in 50 µl aliquots, to the wells. We then incubated the samples for 4 hours at room temperature. Subsequently, we removed the samples and washed the wells four times with 0.05% Tween-20 in PBS. We then added ^{125}I-labelled 19Thy antibody (50,000-100,000 cpm per well) and incubated the wells at 4°C overnight. We then washed the wells four

times and separated each well for bound ^{125}I detection in a Beckman gamma detector.

As shown in Figure 1, in which values were plotted following subtraction for background, the peak fraction of solubilized native T4 protein detected by radioimmunoassay coincided with elution of the 55 Kd protein seen by silver staining.

Western Blot Assay For T4

Although many antibodies have been developed for detecting T4 antigen, none are useful for protein blot analysis (Dr. Ellis Reinherz, personal communication). In order to develop antibodies useful for Western blot detection of soluble T4 to follow the purification of T4 and recombinant soluble T4, we raised polyclonal, hyperimmune anti-T4 antisera in rabbits against three synthetic T4 oligopeptides. These oligopeptides are represented in Figure 3 as follows:

Oligopeptide	Amino Acid Coordinates
JB-1	44-63
JB-2	133-156
JB-3	325-343

We had previously synthesized these peptides using conventional phosphoamide DNA synthesis techniques. See, e.g., Tetrahedron Letters, 22, pp. 1859-62 (1981). We synthesized the peptides on an Applied Biosystems 380A DNA Synthesizer and purified them by gel electrophoresis.

(i) Coupling Of T4 Peptides To BTG

We coupled each of these peptides to the carrier protein bovine thyrogobulin ("BTG") [Sigma, St. Louis, Missouri] according to a modification of procedures set forth in J. Rothbard et al., J. Exp. Med., 160, pp. 208-21 (1984) and R. C. Kennedy et al., "Antiserum To A Synthetic Peptide Recognizes The

HTLV-III Envelope Glycoprotein", Science, 231, pp. 1556-59 (1986).

More specifically, we mixed 10 mg of BTG diluted in 1 ml of PBS with 1.3 mg of m-maleimido-benzoyl-N-hydroxysuccinimide ester ("MBS") in 0.5 ml of dimethylformamide ("DMF"). We mixed the reaction mixture well and reacted it for about 1 hour at 25°C. Subsequently, we loaded the mixture onto a Sephadex G25 gel filtration column (Pharmacia, Sweden) which had been pre-equilibrated with 0.1 M PBS (pH 6.0). We then collected a total of thirty 2 ml aliquot elution fractions and read the absorbance of each fraction at 280 nm ("A_{280}"). We then pooled the three peak fractions (15, 16 and 17) to create the activated carrier.

We dissolved 10 mg of $NaBH_4$ in 2.5 ml of 0.1 M sodium borate solution to produce a sodium borohydride solution. Subsequently, we diluted approximately 8 mg of each of synthetic T4 peptides JB-1, JB-2 and JB-3 with 1 ml of 0.1 M borate buffer and then mixed each solution with 200 µl of the sodium borohydride solution, incubating the mixture on ice for 5 minutes. We then warmed each peptide solution to 25°C, brought each solution to pH 1.0 with 1 N HCl (during which frothing occurred) and then brought each solution to pH 7.0 with 1 N NaOH (after the frothing had stopped).

We then coupled each peptide to BTG by adding 1.2 ml of the peptide solution to 6 ml of the activated carrier solution. We allowed the coupling reaction to proceed overnight by incubating the reaction mixture at room temperature.

(ii) Inoculation Of Test Animals

We dissolved each of the BTG-coupled peptides prepared above in sterile Freund's complete adjuvant, to a final concentration of 1 µg/ml coupled

peptide in PBS. Subsequently, we inoculated each of three rabbits (New Zealand white) by intramuscular injection of 500 µg of one of the coupled peptides into each rabbit. We inoculated a fourth rabbit (New Zealand white) in the same manner with a mixture of the three coupled peptides. All rabbits were prebled prior to boosting to establish an average baseline for each response to be measured. The rabbits were boosted at 6 weeks with 500 µg coupled peptide in incomplete Freund's adjuvant.

Serum was collected from each rabbit monthly for 4 months after immunization. The serum was then assayed for antipeptide titer.

(iii) ELISA With Antipeptide Sera Against Peptide Coated Plates

In this assay, we determined that antiserum raised in an animal by each of peptides JB-1, JB-2 and JB-3 binds to that peptide. Accordingly, those peptides are immunogenic and elicit a response in test animals.

To carry out the assay, we coated Immulon-2 (Dynatech Labs, Alexandria, Virginia) microtiter plates with 50 µl per well of 50 µg/ml uncoupled peptide in PBS and incubated the plates overnight at 4°C. Plates coated with peptide 46R*, which served as controls, were treated identically. We then washed the plates 4 times with PBS-Tween (0.5%) and 4 times with water. The plates were blotted dry by gentle tapping over paper towels. After blotting the plates,

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* Peptide 46 corresponds to amino acids ("AA") 728-751 of the env gene of the HIV genome. The amino acid numbering corresponds to that set forth for the env gene in L. Ratner et al., "Complete Nucleotide Sequence Of The AIDS Virus, HTLV-III", Nature, 313, pp. 277-84 (1985). Peptide 46 has the sequence: LPIPRGPDRPEGIEEEGGERDRDR.

we added 200 µl of a 5% FCS/PBS solution to each well and incubated the plates for 1 hour at room temperature.

We then assayed serum samples from the rabbits on the pre-coated plates prepared as described above. We assayed the antibody response to the immunogen peptide at an initial dilution of 1:100, followed by serial 10-fold dilutions in 5% FCS/PBS.

After a 2 hour incubation period at room temperature, we washed the plates and blotted them dry as described above. We then added 50 µl of a 1:1500 dilution of horseradish peroxidase ("HRP")-conjugated goat anti-rabbit-IgG [Cooper Biomedical, Malvern, Pennsylvania] in 5% FCS/PBS to each well and incubated the plates at room temperature for 1 hour. We washed the plates with PBS-Tween 0.5%. We then added 50 µl of 0.42 mM TMB. We stopped the enzyme reactions with 50 µl of 2 M H_2SO_4. We then analyzed the plates spectrophotometrically at 450 nm using a microtiter plate reader [Dynatech Labs, Alexandria, Virginia].

We observed that antiserum against each of peptides JB-1, JB-2 and JB-3 binds to the corresponding peptide. We also observed that antiserum against a mixture of peptides JB-1, JB-2 and JB-3 binds to peptides JB-1 and JB-3 under the conditions set forth above. The titers of each of the four antisera tested against the peptides in the solid-phase ELISA are shown below, where "ND" represents values not determined:

	Approximate Titer Against:		
Peptide	JB-1	JB-2	JB-3
JB-1	>1/50,000	0	ND
JB-2	0	1/50,000	ND
JB-3	0	0	1/10,000
JB-1 + JB-2 + JB-3	1/4,000	ND	1/7,000

Ig fractions from two of the three anti-peptide sera raised against individual peptides, anti-JB-1 and anti-JB-2, recognized the 55 Kd T4 antigen band of native solubilized T4 in a Western blot analysis of protein eluted from the 19Thy (anti-T4) monoclonal antibody affinity column described above. As in the case of the radioimmunoassay of native solubilized T4, the detection of the 55 Kd protein coincides with its apparent elution from the affinity column. This provides further evidence that our T4 purification procedure enriched for solubilized T4.

Thus, these polyclonal sera are useful in the detection of nanogram quantities of T4 (both native and recombinant forms) by Western analysis.

Binding of Cell-Free T4 To HIV Envelope

We then tested our purified solubilized native T4 isolated from U937 cells for its ability to bind to the HIV envelope protein gp160/gp120. To carry out this direct binding assay, we incubated ^{35}S-labelled gp160/gp120 detergent cell extract derived from a recombinant cell line 7d2 (a gift from Drs. Mark Kowalski and William Haseltine, Dana-Farber Cancer Institute) with samples of solubilized native T4, each of which had been preincubated with one type of monoclonal antibody.

More specifically, we mixed 5 µl of solubilized T4 in a microfuge tube with 5 µg (about 3 µl) of OKT4 (ATCC #CRL 8002), a monoclonal antibody recognizing an epitope on T4 which does not interfere with HIV binding [J. A. Hoxie et al., J. Immunol., 136, pp. 361-63 (1986)] or with 5 µg of OKT4A (Ortho Diagnostics #7142), a monoclonal antibody that interferes with HIV binding to T4 positive cells [J. Steven McDougal et al., J. Immunol., 137, pp. 2937-2944 (1986)]. Alternatively, we mixed 50 µl of solubilized

T4 with 5 µg of αHTLV III gp120 (Dupont #NEN-9284). We then incubated the mixtures on ice for 1 hour.

Subsequently, we added 150 µl of ^{35}S-labelled gp160/gp120 cell extract or ^{35}S-labelled control cell extract (precleared with protein-A Sepharose) to the preincubated solubilized T4/monoclonal antibody mixtures and rocked the tubes overnight at 4°C. We then precipitated the T4/gp160/gp120 immune complexes by adding 30 µl of protein-A Sepharose to each tube and rocking for 2 hours at 4°C to allow the protein-A Sepharose to bind to the antibody complexes. Subsequently, we spun down the beads in an Eppendorf microfuge and after extensive washings, we eluted with 40 µl SDS sample buffer at 65°C for 10 minutes. We then loaded 20 µl of the eluted material on a 7.5% SDS-PAGE gel which was run under reducing conditions.

Figure 2 depicts autoradiograph and Western blot results of the T4/gp160/gp120 coimmunoprecipitations. In Figure 2, lanes 1-5 were autoradiographed after treatment with 40% sodium salicylate and lanes 6-7 were developed on a Western blot with rabbit antisera JB-2.

As shown in Figure 2, gp160/gp120 protein was coimmunoprecipitated in the presence of T4 with OKT4 (lane 5) but not in the presence of T4 with OKT4A (lane 4). Lane 3 shows the positive control for gp160/gp120 using αHTLV III gp120 monoclonal antibody. Neither negative control with ^{35}S-labelled control extract (lane 1) or protein-A Sepharose alone (lane 2) showed bands migrating in the position of gp160/gp120. Based upon the bands that developed on the Western blot, the amount of T4 precipitated with either OKT4 (lane 6) or OKT4A (lane 7) appeared to be similar.

This demonstrates that purified, solubilized native T4, which is naturally membrane bound, can

still interact with the HIV glycoprotein in solution. Accordingly, we believe that cell free soluble T4 is useful in preventing the binding interaction between HIV and the T4 receptor of $T4^+$ lymphocytes. By competing with cell surface T4 for binding to the HIV envelope protein gp120, soluble T4 is useful in blocking HIV infection.

Synthesis Of Oligonucleotide DNA Probes

The nucleotide sequence and a deduced amino acid sequence for a cDNA that purportedly encodes the entire human T4 protein have been reported [Maddon et al., (1985), supra]. The deduced primary structure of the T4 protein reveals that it can be divided into domains as demonstrated below:

Structure/Proposed Location	Amino Acid Coordinates
Hydrophobic/Secretory Signal	-23 to -1
Homology to V-Regions/ Extracellular	+1 to +94
Homology to J-Regions/ Extracellular	+95 to +109
Glycosylated Region/ Extracellular	+110 to +374
Hydrophobic/Transmembrane Sequence	+375 to +395
Very Hydrophilic/ Intracytoplasmic	+396 to +435

Based on the sequence for the above-listed domains, we chemically synthesized antisense oligonucleotide DNA probes using conventional phosphoamide DNA synthesis techniques. See, e.g., Tetrahedron Letters, 22, pp. 1859-62 (1981). We synthesized the probes on an Applied Biosystems 380A DNA synthesizer and purified them by gel electrophoresis.

Furthermore, we synthesized the probes such that they were complementary to the DNA sequences which code for the amino acid sequence, i.e., the probes were antisense, to enable them to recognize and hybridize to the corresponding sequences in DNA, as well as in mRNA. The nucleotide sequences of the eleven selected regions of the T4 protein [corresponding to the nucleotide numbering set forth in Maddon et al., (1985), supra] were the following:

Oligonucleotide	Nucleotide Coordinates
1	145-171
2	742-765
3	1414-1440
6	427-453
7	1303-1329
8	1012-1038
9	97-118
10	10-36
11	1698-1724
12	397-423
14	261-287

Before using our DNA probes for screening, we 5' end-labelled each of the single-stranded DNA probes with ^{32}P using [γ-^{32}P]-ATP and T4 polynucleotide kinase, substantially as described by A. M. Maxam and W. Gilbert, "A New Method For Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-64 (1977).

Construction of λgt10 Peripheral Blood Lymphocytes cDNA Library

To prepare our Peripheral Blood Lymphocytes (PBL) cDNA library, we processed PBL, from a single leukophoresis donor, through one round of absorption

to remove monocytes. We then stimulated the non-adherent cells with IFN-γ 1000 U/ml and 10 μg/ml PHA for 24 hours. We isolated RNA from these cells using phenol extraction [Maniatis et al., Molecular Cloning, p. 187 (Cold Spring Harbor Laboratory) (1982)] and prepared poly A^+ mRNA by one round of oligo dT cellulose chromatography. We ethanol precipitated the RNA, dried it in a speed vac and resuspended the RNA in 10 μl H_2O (0.5 μg/μl). We treated the RNA for 10 min at room temperature in CH_3HgOH (5 mM final concentration) and β-mercaptoethanol (0.26 M). We then added the methyl mercury treated RNA to 0.1 M Tris-HCl (pH 8.3) at 43°C, 0.01 M Mg, 0.01 M DTT, 2 mM Vanadyl complex, 5 μg oligo dT_{12-18}, 20 mM KCl, 1 mM dCTP, dGTP, dTTP, 0.5 mM dATP, 2 μCi[α-^{32}P]dATP and 30 U 1.5 μl AMV reverse transcriptase (Seikagaku America) in a total volume of 50 μl. We incubated the mixture for 3 minutes at room temperature and then for 3 hours at 44°C, after which time we stopped the reaction by the addition of 2.5 μl of 0.5 M EDTA.

We extracted the reaction mixture with an equal volume of phenol:chloroform (1:1) and precipitated the aqueous layer two times with 0.2 volume of 10 M NH_4AC and 2.5 volumes EtOH and dried it under vacuum. The yield of cDNA was 1.5 μg.

We synthesized the second strand according to the methods of Okayama and Berg [Mol. Cell. Biol., 2, p. 161 (1982)] and Gubler and Hoffman [Gene, 25, pp. 263-69 (1983)], except that we used the DNA polymerase I large fragment in the synthesis.

We blunt ended the double-stranded cDNA by resuspending the DNA in 80 μl TA buffer (0.033 M Tris Acetate (pH 7.8); 0.066 M KAcetate; 0.01 M MgAcetate; 0.001M DTT; 50 μg/ml BSA), 5 μg RNase A, 4 units RNase H, 50 μM β NAD , 8 units E.coli ligase, 0.3125 mM dATP, dCTP, dGTP, and dTTP, 12 units T_4 polymerase and incubated the reaction mixture for 90 min at

37°C, added 1/20 volume of 0.5M EDTA, and extracted with phenol:chloroform. We chromatographed the aqueous layer on a G150 Sephadex column in 0.01M Tris-HCl (pH 7.5), 0.1 M NaCl, 0.001 M EDTA and collected the lead peak containing the double-stranded cDNA and ethanol precipitated it. Yield: 0.605 µg cDNA.

We ligated the double-stranded cDNA to linker 35/36:

5'AATTCGAGCTCGAGCGCGGCCGC3'
3' GCTCGAGCTCGCGCCGGCG5'

using standard procedures. We then size selected the cDNA for 800 bp and longer fragments on a S500 Sephacryl column, and ligated it to EcoRI-digested bacteriophage lambda vector gt10 (a gift of Dr. Ellis Reinherz). We packaged aliquots of the ligation reaction in Gigapak (Strategene) according to the manufacturer's protocol. We used the packaged phage to infect E.coli BNN102 cells and plated the cells for amplification. The resulting library contained 1.125×10^6 independent recombinants.

We also screened a PBL cDNA library in the bacteriophage lambda vector gt10 (a gift of Dr. Ellis Reinherz), which was synthesized from mRNA from a $T4^+$ tumor cell line named REX, which expresses T4 protein at high levels [O. Acuto et al., "The Human T Cell Receptor: Appearance In Ontogeny And Biochemical Relationship Of Lambda and Beta Subunits on IL-2 Dependent Clones And T Cell Tumors", Cell, 34, pp. 717-26 (1983)].

Screening Of The Libraries

We then used three of our ^{32}P-labelled synthetic oligonucleotide antisense probes, probes 3, 6 and 9, to screen in parallel our two λgt10 cDNA libraries using the plaque hybridization screening technique described in R. Cate et al., "Isolation Of

The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986), with minor modifications. We modified the Cate et al. procedure by hybridizing without tetramethyl ammonium chloride to accommodate our use of unique probes, rather than mixtures, to probe the plaque filters.

We used the three probes, which had been previously 5' end-labelled with [γ-^{32}P]-ATP according to the method of A. Maxam and W. Gilbert, Meth. Enzymol., 68, pp. 499-80 (1979) to screen in parallel the PBL cDNA library and the REX cDNA library discussed above.

From our screening of the PBL library, we isolated a nearly full length soluble T4 cDNA clone -- λ203-4 (or λgt10.PBL.T4) -- containing a 3.064 kb insert which could be cleaved from the λgt10 vector with EcoRI.

From our screening of the REX cell library, we isolated an incomplete T4 cDNA clone containing a 1,200 bp cDNA insert. We then further characterized the DNA from these clones by DNA sequencing analysis.

We also screened a bacteriophage lambda human genomic library, constructed in the vector EMBL3 by Dr. Mark Pasek (Biogen Inc., Cambridge, Massachusetts) [N. Murray in Lambda 2, eds. R. Hendrix, J. Roberts, F. Stahl, R. Weisberg, pp. 3935-422 (1983)]. The library contains DNA fragments, created by partial restriction of chromosomal DNA from the human lymphoblastid cell line GM1416,48, XXXX (Human Genetic Mutant Cell Repository, Camden, New Jersey) with Sau3a, ligated onto EMBL3 arms which had been subjected to cleavage with BamHI according to the procedures outlined in Maniatis et al., (1982), supra. Plating of the phage library, lysis, and transfer of the phage DNA onto nitrocellulose were performed as described by W. D. Benton and R. W. David, "Screening

of Lambda gt Recombinant Clones By Hybridization To Single Plaques In Situ", Science, 196, p. 180 (1977) and Maniatis et al. (1982). Hybridization conditions were those described by Cate et al. (1986), supra, except that tetramethylammonium chloride (TMACl) was omitted from the washing buffer.

Approximately 2 million plaques were screened in parallel hybridizations with probe 1 and probe 3 discussed above. One phage, called CM47, which hybridized with probe 3 in the primary screenings, was subjected to DNA sequence analysis to determine the existence and position of an intron between the coding sequences for the predicted extracellular and transmembrane domains. No phage clones containing T4 sequences were found screening with probe 1, probably because it includes a sequence interrupted by an intron [D. R. Littman and S. N. Gettner, Nature, 325, pp. 453-55 (1987); and our observations].

Partial sequence analysis of CM47 shows that an intron interrupts the sequence corresponding to the codon for valine (amino acid 363) of the deduced primary sequence for T4 (Figure 3 -- in which introns are indicated by a solid line). This intron defines a potential site for introducing a stop codon in order to express a soluble form of T4. Another intron found within the coding sequence for T4 interrupts the codon for arginine (amino acid 295) and a third intron in CM47 is found between the codons for arginine (amino acid 402) and arginine (amino acid 403) (Figure 3).

Sequencing Of cDNA Clones

We then subcloned EcoRI digested DNA from clone λ203-4 into animal expression vector pBG312 [R. Cate et al., supra] to facilitate sequence analysis. More specifically, as depicted in Figure 4,

we then digested λgt10.PBL.T4 with EcoRI to excise the 3.064 kbp EcoRI-EcoRI fragment containing the full length T4 cDNA. This cDNA sequence, including the entire coding region for soluble T4 and for full length T4 was deposited in p170-2. We used T4 ligase to ligate the fragment into animal expression vector pBG312 [supra] which had been previously cut with EcoRI, to form pBG312.T4 and p170-2 (Figure 4). We then determined the nucleotide sequence of the EcoRI fragment of pBG312.T4 using Maxam Gilbert technology [A. M. Maxam and W. Gilbert, "A New Method For Sequencing DNA", Proc. Natl. Acad. Sci. USA, 74, pp. 560-64 (1977)] (see Figure 3, which depicts the PBL cDNA sequence in comparison to that reported by Maddon et al., (1985), supra). This analysis showed that the 3.064 kbp PBL full length complementary DNA copy of T4 cDNA contained the coding sequence for T4, approximately 200 bp of 5' noncoding sequence and approximately 1500 bp of 3' noncoding sequence.

We then cut pBG312.T4 with PstI and removed the resulting 3' protruding ends with Klenow and isolated an approximately 2.5 kbp fragment. We then inserted the fragment into the polylinker of pBG312 (which had been previously restricted at the SmaI site) to form plasmid p170-2, which contains the full length PBL T4 cDNA sequence (see Figure 3).

As depicted in Figure 3, the PBL T4 cDNA contains a nucleotide sequence almost identical to the approximately 1,700 bp sequence reported by Maddon et al., (1985), supra. The PBL T4 cDNA, however, contains three nucleotide substitutions that, in the translation product of this cDNA, would produce a protein containing three amino acid substitutions compared to the sequence reported by Maddon et al. As shown in Figure 3, these differences are at amino acid position 3, where the asparagine of Maddon et al. is replaced with lysine; position 64,

where the tryptophan of Maddon et al. is replaced with arginine and at position 231, where the phenylalanine of Maddon et al. is replaced with serine. The asparagine reported at position 3 of Maddon et al. instead of lysine was the result of a sequencing error (Dr. Richard Axel, personal communication). The significance of the amino acid replacements at positions 64 and 231, which may represent allellic polymorphism [T. C. Fuller et al., Human Immunology, 9, pp. 89-102 (1982); W. Stohl and H. G. Kunkel, Scand. J. Immunol., 20, pp. 273-78 (1984); N. Amino et al., Lancet, 2, pp. 94-95 (1984); and M. Sato et al., J. Immunol., 132, pp. 1071-73 (1984)], is not known.

DNA sequence analysis [Maxam and Gilbert, supra] of the insert in pEC100 of the REX clone suggests that it represents the product of a splicing error, because 5' noncoding sequence appears to have been spliced with coding sequence beginning with the GGT codon for glycine (amino acid 49) (see Figure 3 and Figure 5). The T4 coding sequence in pEC100* from glycine (amino acid 49) to isoleucine (amino acid 435) is identical to the sequence of Maddon et al., (1985), supra.

In comparison, our earlier N-terminal protein sequence analysis of native T4 protein purified from U937 cells shows a T4 expression product with aspargine as amino acid 3. These differences are also set forth in Figure 6, which also depicts comparisons at corresponding positions of the partial clone from the REX cell line λgt10 library; our

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* We constructed pEC100 by digesting the incomplete T4 cDNA clone from the REX library with EcoRI and isolating the 1,200 bp cDNA insert. We then ligated it to pUC12 (Boehringer Mannheim, Indianapolis, Indiana) which had been previously cut with EcoRI to form pEC100.

genomic clone from a λEMBL3 library; mouse T4 sequences [Tourvieille et al., Science, 234, p. 610 (1986)] and sheep T4 sequences [Classon et al., Immunogenetics, 23, p. 129 (1986)].

Construction of Soluble T4 Mutants

We then employed the technique of in vitro site-directed mutagenesis and restriction fragment substitution to modify the T4 cDNA coding sequence of p170-2 in sequential steps to be identical to that reported by Maddon et al., (1985), supra. We first used oligonucleotide-directed mutagenesis to modify the amino acids at positions 3 and 64. Next, we employed restriction fragment substitution with a fragment including the serine 231 codon of a partial T4 cDNA isolated from a T4 positive lymphocyte cell line [O. Acuto et al., Cell, 34, pp. 717-26 (1983)] library in λgtll (a gift from Dr. Ellis Reinherz), to modify the amino acid at position 231. We then truncated our modified T4 cDNA sequence to remove the coding regions for the transmembrane and intracytoplasmic domains. Subsequently, we constructed three different soluble T4 mutants from our full length T4 clone PBL T4 by linker insertion between restriction sites in order to increase the probability of empirically finding a stable, secretable T4 molecule. The structure of each of these mutants is depicted in Figure 7A.

Line A of Figure 7A represents a hydropathy analysis of our full length soluble T4 carried out using a computer program called Pepplot (University of Wisconsin Genetics Computer Group) according to J. Kyte and R. F. Doolittle, J. Mol. Biol., 157, pp. 105-32 (1982). Line B depicts the protein domain structure of full length T4 [Maddon et al., (1985) supra] in which "S" represents the secretory signal sequence, "V" represents the immunoglobulin-like

variable region sequence, "J" represents the immunoglobulin-like joining region sequence, "U" represents the unique, extracellular region sequence, "TM" represents the transmembrane sequence and "C" represents the cytoplasmic region sequence. In line B, the transmembrane amino acid sequence and some flanking sequence is written below the TM domain. Line C depicts the protein domain structure of recombinant soluble T4 mutants rsT4.1 in pBG377, rsT4.2 in pBG380 and rsT4.3 in pBG381. Line D represents the protein domain structure of E.coli rsT4 gene (Met-perfect construct) (p199-7) which is deleted for the T4 N-terminal signal sequence (S).

We constructed the first three soluble T4 mutant gene fragments by truncating our full length soluble T4 cDNA at positions corresponding to either intron/exon boundaries or to protein domain boundaries defined by hydropathy analysis predictions. More specifically, we introduced synthetic linkers into the unique AvaI site that is 5' to the transmembrane/extracellular domain boundary to produce an in-frame translational stop codon, thus constructing T4 genes that lack the transmembrane and cytoplasmic domains of the full length T4 sequence.

For example, mutant rsT4.1 in pBG377 was truncated by the insertion of a stop codon following amino acid 362, lysine, which corresponds to the position of an intron separating the extracellular and transmembrane domain exons. The positions both of this intron and of the adjacent intron that splits the transmembrane and cytoplasmic domains were determined by DNA sequence analysis of chromosomal T4 clones isolated from the λEMBL3 genomic library described above. Although the significance of the intron positions flanking the T4 transmembrane domain is not known, the determination of the genetic structure could provide important information for design-

ing rsT4 mutants, since exons frequently define functional domains [W. Gilbert, "Why Genes In Pieces?", Nature, 271, p. 501 (1978)].

We then constructed mutant rsT4.2 in pBG380 by truncating the T4 cDNA at the boundary of the transmembrane and extracellular domains at amino acid 374. And, we constructed mutant rsT4.3 in pBG381 by truncating the T4 cDNA at amino acid 377, three amino acids downstream from the transmembrane/ extracellular domain boundary and within the transmembrane domain.

We also employed the technique of oligonucleotide site directed mutagenesis, according to D. Strauss et al., "Active Site Of Triosephosphate Isomerase: In Vitro Mutagenesis And Characterization Of An Altered Enzyme", Proc. Natl. Acad. Sci. USA, 82, pp. 2272-76 (1985), to construct a fourth soluble T4 mutant from our full length T4 clone PBL T4. The structure of this mutant is depicted in Figure 7A, line D, which represents the protein domain structure of E.coli rsT4 gene (Met-perfect rsT4.2) construct, deposited in p199-7, which is deleted for the T4 N-terminal signal sequence (S).

We also constructed various other soluble T4 deletion mutants to determine which smaller fragments of the T4 sequence provide a protein which binds to HIV. These constructions were based on our belief that only the amino terminal sequence of T4 is required for binding to HIV. This belief, in turn, was based upon observations that the monoclonal antibody OKT4A blocks infection of T4 positive cells by HIV and it appears to recognize an epitope in the amino portion of T4 [Fuller et al., supra]. Such fragments of T4, which lack glycosylation and which are capable of binding HIV and blocking infection, may be produced in E.coli or chemically synthesized.

The structure of each of these deletion mutants is depicted in Figure 7B. In that figure, line A depicts the protein domain structure of full length T4 [Maddon et al., (1985), supra; Figure 7A]. In line B, the protein structure of recombinant soluble T4 mutants are depicted as follows: rsT4.7 in p203-5, rsT4.7 in pBG392, rsT4.8 in pBG393, rsT4.9 in pBG394, rsT4.10 in pBG395, rsT4.11 in pBG397, rsT4.12 in pBG396, rsT4.111 in pBG215-7, rsT4.113.1 in pBG211-11 and rsT4.113.2 in pBG214-10.

We constructed soluble T4 derivatives p203-5, pBG392, pBG393, pBG394 and pBG396 by truncating our rsT4.2 gene after the StuI sites at amino acids 183 and 264 of rsT4.2. More specifically, we constructed derivative rsT4.7 in p203-5 and in pBG392 by truncating the rsT4.2 cDNA at amino acid 182. And, we constructed each of derivatives rsT4.9 in pBG394 and rsT4.12 in pBG396 by truncating the rsT4.2 cDNA at amino acids 113, and 166, respectively. One may also construct each of derivatives rsT4.10 in pBG395 and rsT4.11 in pBG397 by truncating the rsT4.2 cDNA at amino acids 131 and 145, respectively.

Expression of T4 and Soluble T4 Polypeptides In Bacterial Cells

The cDNA sequences of this invention can be used to transform eukaryotic and prokaryotic host cells by techniques well known in the art to produce recombinant soluble T4 polypeptides in clinically and commercially useful amounts.

For example, we constructed expression vector p199-7, as shown in Figure 9A, as follows.

We preceded the construction depicted in Figure 9A by the construction of various intermediate plasmids, as depicted in Figures 8A-8D. Those constructions were carried out using conventional

recombinant techniques. The linkers employed in those constructions are set forth in Figure 10.

As depicted in Figures 8A and 8B, starting with p170-2, which contains our full length T4 DNA sequence, coding for T4 characterized by three different amino acids than that of Maddon et al., (1985), supra, we produced various constructs which direct the expression of soluble T4. Some of these constructs are characterized in that one or more of those amino acid differences have been changed to correspond to the respective amino acids of Maddon et al. In this figure, as well as in the other figures, amino acid changes are reflected by an arrow.

Plasmid p192-6 contains the Met perfect rsT4.2 sequence derived by oligonucleotide site-directed mutagenesis which removed the entire T4 N-terminal signal sequence as shown in Figure 8C. And, to provide a convenient means of transferring the rsT4.2 Met perfect sequence into E.coli expression vectors, the steps described in Figure 8D were carried out to produce p195-8, a plasmid containing the Met perfect rsT4.2 sequence flanked by ClaI restriction sites. The ClaI-ClaI cassette of p195-8 optimizes the distance between the 5' ClaI site and the initiating Met codon. In Figure 8D, ST8 rop^- is a tetracycline resistance encoding pAT153-based plasmid containing the rop^- mutation that permits high plasmid copy number, a promoter and ribosome binding site from bacteriophage gene 32 and the gene 32 transcription termination sequence.

Cleavage of p195-8 with ClaI produced the fragment used to assemble p199-7, a construction which directs the expression of Met perfect rsT4.2 under the control of the P_L promoter (Figure 9A). As the first step, to construct a vector from which rsT4.2 expression is under control of the P_L promoter,

we constructed the vector p197-12 from p1034 (plmuGCSF) (Figure 9A).

We then cut p1034 with EcoRI and BamHI to excise the GCSF cDNA insert and a portion of the phage mu ribosome binding site sequence -- which we subsequently reconstructed with oligonucleotides. The synthetic linkers used were linkers 57-60 (Figure 10).

We then ligated the synthetic linker into the EcoRI/BamHI-cut p1034 to form p197-12. One could, instead, replace these steps by starting with any suitable E.coli expression vector containing a ClaI site appropriately placed between the promoter and terminator sequences. We cut p197-12 with ClaI and inserted a ClaI-ClaI cassette containing the cDNA sequence of rsT4.3 in pBG381 and phage transcription terminator derived from p1034. The sequence of this cassette is depicted in Figure 11. The resulting plasmid, p199-7, contains the rsT4.2 "Met perfect" gene in that vector.

Alternatively, one could derive the Met perfect rsT4.2 sequence from plasmid pBG380, deposited in connection with this application, and gap out the signal sequence to create p192-6.

We tested for expression of p199-7 as follows. SG936, an E.coli lon htpr double mutant [ATCC 39624] [S. Goff and A. Goldberg, "ATP-Dependent Protein Degradation In E.coli", in Maximizing Gene Expression, W. Reznikoff and L. Gold (eds.) (1986)], was transformed with p199-7 by conventional procedures [Maniatis et al. (1982)] to form SG936/p199-7, a transformant containing a plasmid with the Met-perfect rsT4.2 gene behind the P_L promoter. Transformants were selected on LB agar plates containing 10 mcg/ml tetracycline (tet). After streaking out several single colonies for single colony isolation, one was chosen at random for testing induction of

rsT4.2 synthesis. We picked a single colony from an LB-agar tet^+ plate into 20 ml Luria Broth (LB) and 10 mcg/ml tet in a 125 ml shake flask and grew it overnight in a shaking air incubator (New Brunswick Scientific, New Jersey) at 30°C.

We then initiated an induction culture by adding 0.5 ml of the overnight culture to 50 ml LB and tet in a 500 ml flask which was grown at 30°C in a shaking air incubator. When the culture reached an OD(600) of 0.4, we transferred it to a 42°C water-bath and shook it gently for approximately 20 minutes. After heat induction at 42°C, the flask was transferred to a 39°C air incubator (New Brunswick Scientific, New Jersey) where it was shaken vigorously at 250 rpm. We withdrew samples just after the 42°C heat shock, and at hourly time points for 4 hours, and then after overnight growth. The samples were measured for growth by OD(600) and analyzed following SDS-PAGE for the pattern of protein synthesis by Coomassie blue protein staining and by Western blot analysis with our rabbit antipeptide antibody probes (described above). Based on the relative molecular weight and protein blot analysis, the expression of rsT4.2 was induced from SG936/p199-7 following heat induction at 42°C (Figure 12).

We transformed p199-7 into a $P_L$mu.tet expression vector, an E.coli expression vector, at the unique ClaI site (see Figure 11). The nucleotide and amino acid sequences of p199-7 are shown in Figure 11.

The expression of soluble T4 from p199-7 in E.coli was measured by Western blot analysis of whole cell extracts following SDS-PAGE using the rabbit polyclonal anti-peptide JB-1 or anti-peptide JB-2 antibodies as probes (Figure 12).

We also constructed expression vector p203-5, as shown in Figure 9B, as follows.

We started with p197-7, which has the same sequence as the $P_L$um vector p197-12 (see Figure 9A), except that there is a single nucleotide deletion in the 5' noncoding region following the P_L promoter. That deletion, which is a deletion of nucleotide #40 -- adenine -- of p197-12 (see Figure 11), resulted from a deletion in the region that was constructed from linkers 57-60 (see Figure 10). p197-7 contains the rsT4.2 gene comprising 374 amino acids. Alternatively, one could also use p197-7 as a starting plasmid.

We cut p197-7 with ClaI. We also cut p195-8 (see Figures 8D and 9A) with ClaI to remove the ClaI - ClaI cassette containing the cDNA sequence of rsT4.2. Subsequently, we inserted the ClaI-ClaI cassette into p197-7 to produce p198-2.

We then digested p198-2 with StuI to remove 80 amino acids (amino acid 185 to amino acid 264) of the mature T4 protein coding sequence. Unexpected methylation, however, prevented cutting at the second StuI site, so that only the StuI site at amino acid 184 was cleaved. Following ligation, the plasmid DNA was transformed into E.coli and we examined several plasmid clones for the deletion using standard procedures. None of those plasmids contained the expected StuI deletion.

Subsequent DNA sequence analysis of one of these plasmids, called p203-5, showed that two guanine residues (see amino acids 183 and 184; nucleotides 818 and 819 of Figure 3) of the StuI recognition sequence had been deleted following cleavage due to exonuclease digestion caused by the use of exonuclease-contaminated StuI enzyme. This dinucleotide deletion produced a translation frameshift following amino acid 182 (glutamine) and introduced a stop codon six amino acid codons downstream from the frameshift (Figure 9C). The unexpected

methylation of the second StuI site together with the deletion that resulted in a new stop codon produced a gene encoding a shortened form of recombinant soluble T4, called rsT4.7. The rsT4.7 sequence encodes a 182 amino acid N-terminal segment of the mature T4 sequence followed by, at the C-terminus, six amino acids -- asparagine-leucine-glutamine-histidine-serine-leucine -- of non-T4 sequence and finally by a TAA stop codon.

The expression of soluble T4 from p203-5 in E.coli was measured by Western blot analysis as previously described.

Expression of T4 and Soluble T4 Polypeptides In Animal Cells

We inserted both soluble T4 genes and the unmodified gene encoding membrane-bound T4 into animal expression vector pBG368. More specifically, we inserted each of the soluble gene constructs into pBG368 under the transcriptional control of the adenovirus late promoter, to give plasmids pBG377, pBG380 and pBG381. We also made two pBG312-based constructions, called pBG378 and pBG379, which direct the expression of recombinant full length T4 protein. pBG378 and pBG379 code for the same full length T4 protein but in pBG379, a portion of the 3' untranslated sequence has been removed. Subsequently, to test for expression of recombinant soluble T4 and recombinant full length T4, we cotransfected Chinese hamster ovary ("CHO") cells with one of each of those plasmids and with the plasmid pAdD26.

We first constructed pBG368 as follows. As depicted in Figure 13, we cut animal cell expression vector pBG312 [R. Cate et al., "Isolation Of The Bovine And Human Genes For Mullerian Inhibiting Substance And Expression Of The Human Gene In Animal Cells", Cell, 45, pp. 685-98 (1986)] with EcoRI and

BglII to delete one of each of the two EcoRI and the two BglII restriction sites (the EcoRI site at position 0 and the BglII site located at approximately position 99). The resulting plasmid, pBG368, retained an EcoRI site in the cloning region and a BglII site after the cloning region. This left a single EcoRI site and a single BglII site in the polylinker for cloning purposes.

More specifically, we deleted one EcoRI site and one BglII site by sequential partial digestion of pBG312 with restriction enzymes EcoRI and BglII, respectively. We filled in with Klenow and 4 nucleotides then religated to produce pBG368, which contains unique restriction sites for EcoRI and BglII enzymes.

Once transient expression of soluble T4 was verified, we constructed stable cell lines that continuously expressed soluble T4. To do this, we employed the stable cell expression host, the dihydrofolate reductase deletion mutant ($DHFR^-$) Chinese hamster ovary cell line [F. Kao et al., "Genetics Of Somatic Mammalian Cells X Complementation Analysis of Glycine-Requiring Mutants", Proc. Natl. Acad. Sci., 64, pp. 1284-91 (1969); L. Chasin and G. Urlab "Isolation Of Chinese Hamster Cell Mutants Deficient In Dihydrofolate Reductase Activity", Proc. Natl. Acad. Sci., 77, pp. 4216-80 (1980)].

Using this system, we cotransfected each T4 gene construct with pAdD26 [R. J. Kaufman and P. A. Sharp, "Amplification And Expression Of Sequences Cotransfected With a Modular Dihydrofolate Reductase Complementary DNA Gene", J. Mol. Biol., 159, pp. 661-21 (1982) containing the mouse DHFR gene. Before carrying out the co-transfections, we linearized all plasmids by restriction enzyme cleavage and, prior to transfection, we mixed each plasmid with pAdD26 so that the molar ratio of pAdD26 to T4

was 1:10. This maximized the number of T4 gene copies per transfectant.

Within the cell, the plasmids were ligated together to form polymers that can become integrated into host chromosomal sequences by illegitimate recombination [J. Haynes and C. Weissmann, "Constitutive, Long-Term Production Of Human Interferons By Hamster Cells Containing Multiple Copies Of a Cloned Interferon Gene", Nucl. Acids Res., 11, pp. 687-706 (1983); S. J. Scahill et al., "Expression And Characterization Of The Product Of A Human Immune Interferon cDNA Gene In Chinese Hamster Ovary Cells", Proc. Natl. Acad. Sci. USA, 80, pp. 4654-58 (1983)]. We selected transfectants that express the mouse DHFR gene in culture medium lacking nucleotides. We then subjected these transfectants to a series of increasing concentrations of methotrexate, a toxic folate analogue that binds DHFR, to select for cells levels of DHFR.

Resistance to methotrexate by increased expression of DHFR is frequently the result of DHFR gene amplification, which can include the reiteration of large chromosomal segments, called amplified units [R. J. Kaufman and P. A. Sharp, "Amplification And Expression Of Loss Of Dihydrofolate Reductase Genes In A Chinese Hamster Ovary Cell Line", Molec. Cell. Biol., 1, pp. 1069-76 (1981)]. Therefore, cointegration of DHFR and rsT4 sequences permitted the amplification of rsT4 genes. Stably transfected cell lines were isolated by cloning in selective growth medium, then screened for T4 expression with a T4 antigen (RIA) [D. Klatzmann et al., Nature, 312, pp. 767-68 (1984)] and by immunoprecipitation from conditioned medium after [^{35}S] cysteine ("^{35}S-Cys") metabolic labelling.

We also inserted the soluble T4 derivative rsT4.7 gene into an animal cell expression plasmid as follows.

As set forth in Figure 14C, we cut plasmid pBG381 (Figure 14A) with EcoRI and NheI. We then cut p186-6 with EcoRI and NheI to remove the 786 base pair fragment. We ligated that fragment into the digested pBG381 to form plasmid pBG391. The T4 sequence in pBG391 is identical to both that of Maddon et al. (1985) supra at positions 64 (tryptophan) and 231 (phenylalanine) and to that of pBG381. However, at position 3, the asparagine reported by Maddon et al. and present in pBG381 is replaced with lysine. The nucleotide sequence of pBG391 is depicted in Figure 15.

We then digested p203-5 with NheI and OxanI to remove the 483 base pair fragment. We inserted that fragment into NheI/OxanI-digested pBG391 to form plasmid pBG392, the animal cell expression construct of rsT4.7. The T4 sequence in rsT4.7 contains amino acids identical to that of Maddon et al.'s full length sequence at amino acid positions 64 (tryptophan) and 231 (phenylalanine). However, at position 3, the asparagine reported by Maddon et al. is replaced with lysine. The nucleotide sequence of pBG392 is depicted in Figure 16.

In Figure 14D, we have depicted the construction of other animal cell expression constructs containing sequences encoding the deletions rsT4.9 in pBG394, and rsT4.12 in pBG396. Those constructions were carried out using conventional recombinant techniques. The linkers employed in those constructions are set forth in Figure 18. The nucleotide sequences of pBG394 and pBG396 are shown in Figures 19 and 20.

Plasmid pBG393, shown in Figure 17, contains rsT4.8, the perfect form of rsT4.7. pBG393 contains 182 amino acids of the mature T4 sequence, without the additional non-T4 6 amino acids at the C-terminus following amino acid 182. The nucleotide sequence of BG393 is shown in Figure 21.

Other animal cell expression plasmids according to this invention may be constructed as depicted in Figure 17. These include rsT4.10 in pBG395 and rsT4.11 in pBG397 (see Figure 18 for specific linkers).

The nucleotide sequence of BG395 is shown in Figure 22.

Purification Of Recombinant Soluble T4

Recombinant soluble T4 construct pBG380 expressed in DHFR$^-$ CHO cells was grown to confluency in a α-Modified Eagles Medium (Gibco) supplemented with 10% fetal calf serum, 1 mM glutamine and the antibiotics penicillin and streptomycin (100 µg/ml of each). The cells were grown at 37°C in two 21 Cell Factory Systems (Nunc). We then washed the confluent cells free of fetal calf serum with α-Modified Eagles Medium without fetal calf serum and cultured the cells in α-Modified Eagles Medium at 37°C for 4 days. Subsequently, we harvested the conditioned media, filtered it through a Millipore Millidisk 0.22µ hydrophilic filter cartridge (Millipore #MCGL 305-01) and concentrated the secreted proteins on a fast-S ion exchange column (S-Sepharose Fast Flow, Pharmacia #17-0511-01) in 20 mM MES buffer (pH 5.5).

We then eluted the bound proteins with 20 mM Tris-HCl (pH 7.7) and 0.3 M NaCl. The elution pool was subsequently diluted with 2 volumes of 20 mM Tris-HCl (pH 7.7) and it was then loaded on a column comprising immobilized 19Thy anti-T4 monoclonal antibody coupled to Affigel-10 [a gift of Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, Massachusetts]. We washed the column extensively and eluted the bound material as 0.5 ml fractions with 50 mM glycine-HCl (pH 2.5), 150 mM NaCl, 0.1 mM EGTA and 5 µg/ml bovine pancreatic trypsin inhibitor, Aprotinin (Sigma #A1153). We used Western blots

developed with rabbit antisera raised against peptide JB-2 to follow the purification. We employed silver stained gels to follow binding and elution of rsT4.2 during the chromatography. Figure 23 depicts a Coomassie stained gel of purified rsT4.2.

Gel sizing-column chromatography analysis of the purified rsT4.2 from the pBG380 transfected CHO cell line, BG380G, suggests that rsT4 is monmeric under physiologic pH and salt concentration.

Sequencing Of Recombinant Soluble T4 Protein

We then determined the N-terminal amino acid sequence of a recombinant soluble T4, specifically rsT4.2, molecule purified from the conditioned medium of the pBG380 transfected CHO cell line BG80G, as described above, by automated Edman degradation in an Applied Biosystems 470A gas phase sequenator [R. B. Pepinsky et al., J. Biol Chem., 261, pp. 4239-46 (1986)].

The amino terminal sequence matched the sequence which we had previously determined for solubilized native T4 isolated from U937 cells, supra. The amino terminal sequences of native solubilized T4 (sT4) and purified rsT4 protein are Δ2 proteins, as compared to the amino terminal sequence predicted by Maddon et al., (1985), supra, with the mature amino terminus located at position 3 of that sequence. The amino terminal sequences of solubilized native T4 (sT4), recombinant soluble T4 (rsT4.2) secreted by CHO transfectant BG380G containing pBG380 and the protein sequence deduced by Maddon et al. (1985), supra are as follows:

sT4: X-K-V-V-L-X-K-K-X-D-T-V-E-L-T-X-T-A-S-E-

rsT4.2: N-K-V-V-L-G-K-K-G-D-T-V-E-L-T-X-T-A-S-E-

Maddon et al. Q-G-N-K-V-V-L-G-K-K-G-D-T-V-E-L-T-C-T-A-S-E

In the above sequences, the amino acids are represented by single letter codes as follows:

Phe:	F	Leu:	L	Ile:	I	Met:	M
Val:	V	Ser:	S	Pro:	P	Thr:	T
Ala:	A	Tyr:	Y	His:	H	Gln:	Q
Asn:	N	Lys:	K	Asp:	D	Glu:	E
Cys:	C	Trp:	W	Arg:	R	Gly:	G

X: not determined or ambiguous.

We also constructed pBG211-11, a plasmid coding for the N-terminal 113 amino acids of soluble T4 protein. This construct, which codes for a protein characterized by a single disulfide bridge, between the cysteines at amino acid positions 18 and 86, is conveniently expressed in E.coli.

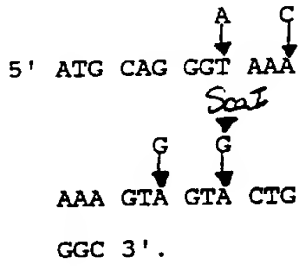
To construct p211-11, as depicted in Figure 24, we first cut p195-8 (see Figures 8D and 9A) with ClaI to remove the ClaI-ClaI cassette containing the cDNA sequence of rsT4.2. We then digested pAT153γ3SH16ΔAmp, the tryptophan operon promoter plasmid from the gamma interferon producing E.coli strain BN374 with ClaI, and deleted the cDNA coding for gamma interferon. Subsequently, we inserted the ClaI-ClaI cassette into the ClaI-cut E.coli plasmid in front of the tryptophan operon promoter and ligated to produce p196-10.

As shown in Figure 25, we then subjected pBG380 to oligonucleotide-directed mutagenesis to insert three tandem translational stop codons following the T4 cDNA sequence coding for amino acids -23 to 113 in pBG380, to produce pBG394.

We then constructed p211-11 from fragments of each of p196-10, pBG394 and p1034 as depicted in Figure 26. The first fragment including the vector sequences, was produced by restricting p196-10 with

HindIII and ClaI to remove the T4 coding sequence from amino acids 61 through 374 of rsT4.2 and including vector sequence following the 3' end of the rsT4 gene. The second fragment, a HindIII - BglII segment including the codons for T4 amino acids 61-113 of rsT4.9 immediately followed by a triplet of stop codons in tandem, was isolated by HindIII/BglII digestion of pBG394. The third fragment, a BamHI - ClaI fragment containing a bacteriophage T4 transcriptional termination signal [H. N. Kirsch and B. Allet, "Nucleotide Sequences Involved In Bacteriophage T4 Gene 32 Translational Self-Regulation", Proc. Natl. Acad. Sci. USA, 79, pp. 4937-41 (1982)], was isolated by BamHI/ClaI digestion of p1034. We then ligated these three fragments to produce p211-11, a T4 construct coding for a 113 amino acid soluble form of T4 protein, with asparagine at amino acid position 3 (i.e., rsT4.113.1).

We then subjected p211-11 to oligonucleotide site-directed mutagenesis (Figure 27) to change the amino acid at position 3 from asparagine to lysine using the oligonucleotide T4-66:



This produced plasmid p214-10, a fully corrected 113 amino acid soluble T4 vector coding for a 113 amino acid soluble form of T4 protein, with lysine at amino acid position 3 (i.e., rsT4.113.2). As shown in Figure 27, we subjected p214-10 to oligonucleotide site-directed mutagenesis to delete glutamine and glycine at, respectively,

amino acid positions 1 and 2 of the T4 sequence using the oligonucleotide T4AID-87:

```
          C
          ↓
5' GTA TCG ATT TGG
   ATG ATG AAA AAA
   GTA GTA 3'.
```

This produced p215-7, a 111 amino acid soluble T4 construct, including the trp promoter, which directs the expression of a 111 amino acid soluble form of T4 protein, with lysine at amino acid position 3 (i.e., rsT4.111).

We next constructed p218-8, a 111 amino acid construct which directs the expression of a 111 amino acid soluble form of T4 protein, with lysine at amino acid position 3 (i.e., rsT4.111) under the control of the P_L promoter, as depicted in Figure 28.

More specifically, we cut p197-12 (Figure 9A) with ClaI to remove the 101 bp fragment containing linker and terminator sequences. We also cut p215-7 with ClaI to remove the ClaI - ClaI cassette containing the cDNA sequence of rsT4.111 and the ϕT4 transcriptional terminator sequence [Kirsch and Allet, supra]. Subsequently, we inserted the ClaI - ClaI cassette into the ClaI-cut p197-12 to produce p218-8.

In order to express rsT4.113.1, we transformed E.coli A89 with p211-11 by conventional techniques [Maniatis et al. (1982), supra] to form E.coli A89/p211-11. E.coli A89 is a tetracycline sensitive derivative of E.coli SG936. We isolated E.coli A89 from E.coli SG936 according to the method of S. R. Maloy and W. D. Nunn, "Selection For Loss Of Tetracycline Resistance By Escherichia coli", J. Bact., 145, pp. 110-12 (1981), which is based upon the ability of the lipophilic chelating agent fusaric acid to selectively inhibit resistant strains.

More specifically, we plated E.coli SG936 on medium containing, per liter, 5 g tryptone, 5 g yeast extract, 10 g NaCl, 10 g $NaH_2PO_4 \cdot H_2O$, 50 mg chlortetracycline-HCl, 12 mg fusaric acid, 0.1 mM $ZnCl_2$ and 15 g agar. Colonies which grew at 30°C (putative tetracycline-sensitive strains) were retested for tetracycline sensitivity on L-agar plates containing 5 µg/ml tetracycline. One tetracycline-sensitive strain, designated A89, was then shown to be unable to grow on LB agar at 42°C, thus verifying the presence of the htpR mutation.

Transformants were selected by tetracycline resistance. We picked a single colony into 20 ml of minimal medium plus 0.2% casamino acids plus tryptophan (100 µg/ml) plus tetracycline (10 µg/ml) in a 100 ml shake flask placed in a shaking air incubator at 30°C and allowed the cells to grow up overnight. The following morning, we inoculated 40 ml of minimal medium plus 0.2% casamino acids plus tryptophan (100 µg/ml) plus tetracycline (10 µg/ml) with the overnight culture at OD_{600} = 0.05 in a 500 ml flask. The cells were grown to midlog phase and then induced by pelleting, washing once in minimal medium and then resuspending in minimal medium plus 0.2% casamino acids plus tetracycline (10 µg/ml), in the absence of tryptophan. We removed 0.6 OD_{600} of cells after 0, 1, 2, 3 and 4 hours incubation and after growth overnight.

The aliquots were centrifuged and cell pellets were subjected to lysis by boiling in Laemmli gel loading buffer. After centrifugation to remove cell debris, half of each sample was subjected to SDS-PAGE, followed by Western blot analysis with our rabbit antipeptide antibody probes or by Coomassie blue protein staining (Figures 29A and 29B).

Purification Of rsT4.113.1

We then purified rsT4.113.1 from the E.coli transformant by means of two essentially quantitative steps involving anion-exchange and gel-filtration chromatographies performed under reducing and denaturing conditions.

More specifically, we suspended 14 g of wet cells from a 4 L shake-flask fermentation in 100 ml of a 20mM Tris (pH 7.5) buffer containing 20 µg/ml DNase, 20 µg/ml RNase and 1 mM phenylmethylsulfonylfluoride ("PMSF"). The suspension was applied to a French Press at 1000 psi in two passages and then centrifuged in an SA 600 rotor at 18,000 g for 15 min at 4°C. The resulting pellet was solubilized in 20 ml of a 20 mM Tris (pH 7.5) buffer containing 7 M urea and 10 mM 2-mercaptoethanol. We then subjected the suspension to ultracentrifugation at 85,000 g for 90 min at 4°C. The supernatant was diluted by the addition of 80 ml of 20 mM Tris (pH 7.5) buffer containing 7 M urea and 10 mM 2-mercaptoethanol and 40 ml of the sample was applied to a 3 x 4 cm Q-Sepharose fast-flow column (Sigma, St. Louis, Missouri) which had been pre-equilibrated in the same buffer. The column was developed with a gradient in 400 ml total volume of increasing NaCl from 0 to 0.3 M in the same Tris/urea/2-mercaptoethanol buffer. Column fractions were monitored for absorbance at 280 nm and for protein content by SDS-PAGE (15% acrylamide). The fractions were also analyzed by Western blots. Figure 30, panel (a) is a chromatogram displaying the purification of rsT4.113.1 by ion-exchange chromatography. In that figure, peaks containing rsT4.113.1 are identified. The rsT4.113.1 was found to elute early in the NaCl gradient and to be well-resolved from low-molecular weight contaminants.

In order to separate rsT4.113.1 from high-molecular weight contaminants, we carried out gel-filtration chromatography on an rsT4.113.1-containing pool for final purification of the protein to near homogeneity (>95% purity). More specifically, we prepared a pool containing 20 mg of protein in 50 ml and then concentrated to 10 ml in a stirred-cell ultrafiltration unit (Amicon, Danvers, MA.) using a PM-30 membrane (Amicon). Subsequently, 5.0 ml of the concentrate was applied to a 1.5 x 95 cm S-300 column (Sigma) equilibrated and developed in the same Tris/urea/2-mercaptoethanol buffer. We monitored the column fractions for absorbance at 280 nm and for protein content by SDS-PAGE. The fractions were also analyzed by Western blots. A pool containing rsT4.113.1 (approximately 4 mg) in 15 ml was thus prepared. Figure 30, panel (b) is a chromatogram displaying the purification of rsT4.113.1 by gel-filtration separation of the rsT4.113.1 pool. In that figure, peaks containing rsT4.113.1 are identified.

Figure 30, panel (c) is an SDS-PAGE analysis depicting the purification of the rsT4 derivative throughout the centrifugation and chromatography steps. In Figure 30, panel (c), the lanes depicted are:

lane A: molecular weight standards
lane B: cell extracts
lane C: cell pellet following solubilization of cell extract in non-denaturing conditions
lane D: supernatant following solubilization of cell extract in non-denaturing buffer
lane E: supernatant following ultracentrifugation step

lane F: Q-Sepharose pool
lane G: S-300 gel-filtration pool.

Refolding Of Purified rsT4.113.1

We refolded the purified rsT4.113.1 by dilution and dialysis steps to non-denaturing and oxidized conditions. More specifically, refolding of the protein at a concentration of 0.5 OD (280)/ml was achieved by stepwise dialysis against 500 volumes of 3 M urea, 20 mM Tris (pH 7.5); 500 volumes of 1 M urea, 0.1 M ammonium acetate (pH 6.8) and, finally, the same volume of a phosphate-buffered saline solution. Throughout the refolding procedure, samples of the protein were monitored for relative content by spectral analysis and by high-performance liquid chromatography ("HPLC") performed on a 150A liquid chromatographic system (Applied Biosystems, Inc., Foster City, California). An octasilyl column (Aquapore RP-300, 0.46 x 3.0 cm) was equilibrated in 80% 0.1% trifluoroacetic acid ("TFA")/water (solvent A) and 20% 0.085% TFA/70% acetonitrile (solvent B) and developed with a linear gradient of increasing acetonitrile concentration from 20% to 80% (solvent B) over 45 min at a flow rate of 0.5 ml/min.

As shown in Figure 31, panel (a), protein in 7 M urea, 10 mM 2-mercaptoethanol and 20 mM Tris(pH 7.5) eluted from the HPLC column at 49% acetonitrile in the gradient. In subsequent steps, from 1 M urea/1 mM ammonium acetate (pH 6.8) [Figure 31, panel (b)] to phosphate buffered saline [Figure 31, panel (c)], an increasing percentage of rsT4.113.1 was found to elute earlier in the HPLC gradient -- at 47% acetonitrile. The identity of the earlier eluting peak as oxidized product was verified by reduction of rsT4.113.1 in non-chaotropic

solutions and application of sample thus treated to HPLC under the same conditions.

The elution of oxidized rsT4.113.1 prior to reduced protein on HPLC suggests that formation of the single disulfide bridge decreases relative hydrophobicity of the protein [J. L. Browing et al., Anal. Biochem., 155, pp. 123-28 (1986)]. Spectral analysis of rsT4.113.1 was performed throughout the course of refolding in order to monitor relative yield of soluble protein in the procedure. The refolding method allowed approximately 20% recovery of rsT4.113.1. HPLC analysis indicated a less than 15% contaminant of reduced protein in the preparation (Figure 30, panel (c), lane G).

Sequencing Of Renatured rsT4.113

We then carried out amino acid analysis of rsT4.113.1 by automated Edman degradation in an Applied Biosystems 470A gas phase sequenator equipped with a 900 A data system. Phenylthiohydantion amino acids generated during the course of the degradative chemistry were analyzed on-line using an Applied Biosystems 120A PTH-analyzer equipped with a PTH-C18 2.1 x 220 mm column. Protein (10 µg) for sequence analysis was applied to SDS-PAGE (15% acrylamide) and electroblotted on an Immobilon membrane (Millipore Corp., Bedford. Massachusetts) as described by P. Matsudaira, J. Biol. Chem., 262, pp. 10035-38 (1987).

Amino acid analysis of protein samples was performed by hydrolysis of protein in 6 N HCl, in vacuo, for 24 h at 110°C. The hydrolysates were then applied to a Beckman 6300 Analyzer equipped with post-column detection by ninhydrin. Western blot analysis of the SDS-PAGE gels was carried out by standard techniques using rabbit antisera JB-1.

Sequence analysis revealed an amino terminal sequence of: Met-Gln-Gly-Asn-Lys-Val-Val ...

The purified rsT4.113.1 protein was found to contain stoichiometric quantities of the amino-terminal methionine placed in the protein construct for expression in E.coli and an intact polypeptide chain consistent with a sequence derived from the plasmid construction. Recovery of phenylthiohydantoinyl-methionine at the first cycle of the degradative chemistry was 60% consistent with routine initial yields obtained in the automated Edman. This observation excludes the possibiity that a significant percentage of the rsT4.113.1 lacked the initiation methionine, i.e., the NH_2-methionine was not removed by expression of rsT4.113.1 in E.coli, or that sequence analysis was impaired by the presence of glutamine at the first cycle of the degradative chemistry. Sequence analysis was performed for 40 cycles and no evidence of lysine carbamylation was observed. Amino acid analysis displayed a close correlation of actual and theoretical values for amino acids, thus indicating the marked absence of proteolytic degradation in the course of expression, or purification, or both.

Immunoprecipitation Of CHO Cell Lines Producing Soluble T4

We tested the conditioned media from ^{35}S-Cys metabolically labelled CHO cells transfected with one of the T4 mutant constructs pBG377, pBG380, pBG381, the full length recombinant T4 construct pBG379, of this invention or vector only, to determine whether any produced a molecule recognized by the anti-T4 monoclonal antibody 19 Thy. To carry out this test, we incubated about 10^7 CHO cells transfected with either pBG380, pBG381, pBG377, pBG379 or pBG312, for 5 hours at 37°C with 180 μCi/ml ^{35}S-labelled cysteine

[DuPont, New England Nuclear] in 4 ml RPMI cys^- medium (Gibco). After labelling of the cells, 1 ml of filtered, conditioned media was made 0.5 mM with phenyl-methyl-sulphonyl fluoride and immunoprecipitated with OKT4 and protein A Sepharose [P. H. Sayre and E. L. Reinherz, Eur. J. Immunol., 15, pp. 291-95 (1985)]. Subsequently, we incubated media from the ^{35}S-labelled cells with OKT4 (ATCC #CRL 8002). We then immuno-precipitated with protein A Sepharose and subjected the immuno-precipitates to SDS-PAGE under reducing conditions on 10% polyacrylamide gels [U. K. Laemmli, Nature, 227, pp. 680-85 (1980)]. Autoradiography was carried out with X-Omat X-ray film (Eastman Kodak).

As shown in lanes 3-5 of Figure 32, both pBG380 (rsT4.2) and pBG381 (rsT4.3) directed the synthesis of a secreted, immune, ^{35}S-labelled T4 protein that was recognized by the OKT4 anti-T4 antibody. The immunoprecipitated truncated molecules migrated as 49 Kd proteins, a result consistent with their predicted molecular weights. In contrast, no soluble T4 antigen could be detected in the conditioned media of cell lines stably transfected with pBG377 (rsT4.1) or pBG379 (rflT4). Immunoprecipitation analysis of cellular extracts of cell lines transfected with pBG377 suggests that the rsT4.1 gene may be misfolded, which could account for a block in its secretion [M. J. Gething et al., Cell, 46, pp. 939-50 (1986)].

In Figure 32, the lanes represent the following: Lane 1: immunoprecipitation from conditioned medium of CHO cells stably co-transfected with vectors pBG312 and pAdD26. Lane 2: blank. Lanes 3 and 4: immunoprecipitation from conditioned medium of CHO cells stably co-transfected with pBG380 (rsT4.2) and pAdD26. Lanes 5 and 6: immunoprecipitation from conditioned medium of CHO cells stably

co-transfected with pBG381 (rsT4.3) and pAdD26. Lane 7: immunoprecipitation from conditioned medium of CHO cells stably co-transfected with recombinant full length T4 (pBG379) and pAdD26. In Figure 32, the arrow indicates the predicted position of the soluble T4 from pBG380 or pBG381 relative to the migration of standard molecular weight markers.

Immunoprecipitation Of COS 7 Cell Lines Producing Recombinant Soluble T4

We expressed recombinant soluble T4 derivatives pBG392, pBG393 and pBG394 in COS 7 cells by electroporation, essentially as described by G. Chu et al., "Electroporation For The Efficient Transfection Of Mammalian Cells With DNA", Nuc. Acids Res., 15, pp. 1311-26 (1987). More specifically, we introduced 20 µg closed circular plasmid DNA and 380 µg of carrier (sonicated salmon sperm DNA) into 3 x 10^7 COS 7 cells. The cells were electroporated using a Gene Pulser (Biorad) set at 300 volts. Subsequently, we incubated the COS 7 cells in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum for 24 hours. We then harvested the conditioned media, filtered it through a Millipore Millidisk 0.22 µ hydrophilic filter cartridge (Millipore #MCGL 305-01) and concentrated the secreted proteins on a fast-S ion exchange column (S-Sepharose Fast Flow, Pharmacia #17-0511-01) in 20 mM MES buffer (pH 5.5).

We then eluted the bound proteins with 20 mM Tris-HCl (pH 7.7) and 0.3 M NaCl. The elution pool was subsequently diluted with 2 volumes of 20 mM Tris-HCl (pH 7.7) and it was then loaded on a column comprising either 19Thy anti-T4 monoclonal antibody and protein A Sepharose or OKT4A and protein A Sepharose. We washed the column extensively and eluted the bound material as 0.5 ml fractions with

50 mM glycine-HCl (pH 2.5), 150 mM NaCl, 0.1 mM EGTA and 5 μg/ml Bovine pancreatic trypsin inhibitor, Aprotinin (Sigma, #A1153). The immunoprecipitates were subjected to SDS PAGE (10% gel) followed by immunoblotting against rabbit antisera raised against peptide JB-1. We employed silver stained gels to follow binding and elution of rsT4 during chromatography.

Figure 33 depicts an immunoblot analysis of transiently expressed pBG392 (rsT4.7) [lanes 10, 11]; pBG393 (rsT4.8) [lanes 4, 7, 8] and pBG394 (rsT4.9) [lane 5]. The standards are 50 ng purified rsT4.3 (lane 1); 150 ng purified rsT4.3 (lane 2) and 250 ng purified rsT4.3 (lane 3). The arrow indicates the expected position of migration of a protein with the relative molecular weight of rsT4.7: 21,000 daltons. The sample that was to be loaded into lane 4 was lost and lanes 6 and 9 are blank.

As shown in lanes 10 and 11 of Figure 35, pBG392 (rsT4.7) directed the synthesis of a secreted, immune protein that was recognized by the anti-T4 antibodies OKT4A and 19Thy. Lanes 4, 7 and 8 also demonstrate that pBG393 (rsT4.8) directed the synthesis of a secreted, immune protein that was recognized by OKT4A and 19Thy. This analysis illustrates that rsT4.7 contains the OKT4A epitope. It also suggests that the binding region for HIV envelope binding resides in the amino 182 terminal residues of T4.

In contrast, no soluble T4 could be detected in the media of cell lines transfected with pBG394 (rsT4.9) [see lane 5]. Immunoprecipitation analysis of cellular extracts of cell lines transfected with pBG397, however, showed that rsT4.9 was recognized by OKT4A. We believe that rsT4.9, a 113 amino acid construct, binds the HIV virus and that it represents a second generation soluble T4, one with only two

cysteines and one of three disulfide bridges. Accordingly, rsT4.9 is easily produced in E.coli or yeast systems.

Similarly, although no soluble T4 could be detected in the media of cell lines transfected with pBG396 (rsT4.12), analysis of cellular extracts of those cell lines showed that rsT4.12 was recognized by OKT4A. Thus, rsT4.12 may also bind HIV virus.

Radioimmunoassay And Epitope Analysis Of rsT4.113

In order to determine if the 113 fragment of rsT4 contained structural determinants for binding to OKT4A, Leu-3A and OKT4, we then carried out radioimmunoassay and epitope analysis of rsT4.113 using a competitive inhibition radioimmunoassay [C. J. Newby et al., "Solid-Phase Radioimmune Assays" in Handbook Of Experimental Immunology, D. M. Weir (Ed.), 1, pp. 34.1-34.8 (1986)]. As OKT4A and Leu-3A block infectivity of HIV in vitro [Dalgleish et al., supra] and binding of T4 to gp120/160 [McDougal et al., supra], this analysis served as a first approximation as to whether or not rsT4.113 contained structural elements for interaction with HIV.

We first coated U-bottom 96 well microtiter plates (Falcon) with 50 µl/well goat-anti-mouse IgG (Hyclone Typing Kit, Logan, Utah) in PBS (pH 7.0) to a concentration of 50 µg/ml and incubated the plates overnight at 4°C. We then rinsed the plates with 1X PBS and blotted them dry. The plates were then blocked by the addition of 100 µl/well of a 1X PBS solution containing 5% bovine serum albumin for 1 hour at room temperature. We rinsed the plates with PBS, blotted dry and then spotted them with 50 µl of one of three antibody solutions containing either OKT4 (10 µg/ml in block buffer); OKT4A (500 ng/ml in block buffer) or Leu-3A (Becton-

Dickinson) (500 ng/ml in block buffer). We let the plates stand for 2 hours at room temperature. We then washed the plates 3 times with a PBS/0.05% Tween-80 solution and 2 times with 1X PBS and blotted them dry.

In a separate plate, we titrated competitor samples of unlabeled rsT4.113.1 from 20 µg/ml and serially diluted twice (including no competitor control), with final volumes in each well of 25 µl. The positive control for this assay was competition with unlabeled rsT4.3 (375 amino acids). We then added 25 µl of ^{125}I-rsT4.3 containing 10,000 cpm/25 µl (prepared according to A. E. Bolton and W. M. Hunter, Radioimmunoassay And Related Methods, Chapter 2c). Subsequently, we spotted the entire 50 µl content of each well onto the assay plate containing each of the three antibody solutions and incubated for 2 h at room temperature. We then washed the plates 3 times with a PBS/0.5% Tween-80 solution and 2 times with 1X PBS, blotted them dry and then counted the wells in a Beckman gamma counter for radioactivity.

As shown in Figure 34, rsT4.113.1 competes with ^{125}I-rsT4.3 for absorption to an OKT4A solid phase in a dose-dependent manner. Additionally, rsT4.113.1 competes with ^{125}I-rsT4.3 for absorption to a Leu-3A solid phase in a dose-dependent manner. By comparison to unlabeled rsT4.3, rsT4.113.1 exhibits a molar affinity for those antibodies within a factor of 3. In the 0.4 to 25 µg/ml concentration range tested, rsT4.113 did not compete with radiolabelled rsT4.3 for binding to OKT4. In a similar assay, we observed that rsT4.111 also competes with ^{125}I-rsT4.3 for binding to OKT4A and Leu-3A, but not to OKT4 [Figures 35-37].

Based on these results, we believe that the epitopes for OKT4A and Leu-3A are contained within

the amino-terminal 113 amino acids of T4. We also believe that the epitope for OKT4 binding is localized within the carboxy terminal of the T4 polypeptide.

Accordingly, we believe that the gp120-binding domain is localized within the amino terminal 113 or 111 amino acids of the T4 protein. Based on this belief, we synthesized various synthetic oligopeptides which contain sequence within that structural domain. These oligopeptides are represented in Figure 3 as follows:

Oligopeptide	Amino Acid Coordinates
JB-1	44-63
rsT4 #6	18-29
rsT4 #7	5-56
rsT4 #8	84-97
rsT4 #9	30-63

We synthesized these peptides using conventional phosphoamide DNA synthesis techniques [Tetrahedron Letters, 22, pp. 1859-62 (1981)]. We synthesized the peptides on an Applied Biosystems 380A DNA Synthesizer and purified them by gel electrophoresis.

ELISA Assay For rsT4.113

We also carried out an ELISA assay for rsT4.113.1 produced by p211-11-transformed E.coli. Throughout this assay, dilutions were made in blocking solution and, between each step, we washed the plates with PBS/0.05% Tween-20. More specifically, we coated wells of Immulon 2 (Dynatech, Chantilly, Virginia) plates with .005 OD (280 nm)/ml of OKT4 (IgG2b) in 0.05 M bicarbonate buffer to a volume of 50 µl/well and incubated the plates overnight at 4°C. We then blocked the plates with 5% bovine serum albumin in PBS, 200 µl/well, and incubated for 30 minutes at room temperature.

Subsequently, we added 50 µl of 50 ng/ml rsT4.3 to each well, incubating overnight at 4°C.

We then added 50 µl/well of a mixture containing rsT4.113.1 and 10 ng/ml of OKT4A and incubated for 2 1/2 hours at room temperature. Using a Hyclone Kit (Hyclone), we then carried out the following steps. First, we added 1 drop of rabbit anti-mouse IgG2a to each well and incubated the plates for 1 hour at room temperature. We then added 100 µl of peroxidase-labeled anti-rabbit IgG, diluted 1:4000 with blocking buffer to each well, and incubated for 1 hour at room temperature.

We prepared a substrate reagent as follows. We diluted substrate reagent 1:10 in distilled water and added two O-phenyl-ethylene-diamine ("OPD") chromophore tablets per 10 ml of substrate. We let the mixture dissolve thoroughly by mixing with a vortex. Alternatively, a TMB peroxidase substrate system (Kirkegaard & Perry Catalogue #50-76-00) may be used. Subsequently, we added 100 µl of the chromophore solution to each well, incubated for 10-15 minutes at room temperature and then stopped the color development with 100 µl of 1N H_2SO_4. We then measured OD at 490 nm, using an ELISA plate reader.

The results of the assay are demonstrated in Figure 38.

We then subjected the soluble T4 proteins produced by the T4 constructs of this invention to various functional assays.

Assays Of The Antiviral Activity Of Soluble T4

The antiviral activity of soluble T4 according to this invention was evaluated using modifications of various in vitro systems used to study antiviral agents and neutralizing antibodies [D. D. Ho et al., "Recombinant Human Interferon Alpha (A) Suppresses HTLV-III Replication In Vitro", Lancet, pp. 602-04 (1985); K. Hartshorn et al.,

"Synergistic Inhibition Of HTLV-III Replication In Vitro By Phosphonoformate And Recombinant Interferon Alpha-A", Antimicrob Ag Chemoth, 30, pp. 189-91 (1986)].

For each of these assays, we prepared graded concentrations of soluble T4 and preincubated them with an H9 derived IIIB isolate of HIV [a gift from Drs. M. Popovic and R. Gallo, National Cancer Institute, Bethesda, Maryland]. The isolate was maintained as a chronically infected culture in H9 cells. Cell-free HIV stocks were obtained from supernatant fluids of HTLV-III infected H9 cultures (culture conditions: 1 x 10^6 cells/ml with 75% viable cells). We prepared serial 10 fold dilutions of recombinant soluble T4 ranging from 10 picograms/ml to 10 micrograms/ml and incubated them with fifty 50% tissue culture infectious doses ($TCID_{50}$) of HIV for 1 hour at 37°C, in RPMI-1640 supplemented with 20% heat inactivated fetal calf serum (FCS). We then added 150 µl of H9 cells to a final concentration of 0.5 x 10^6 cells/ml which were not HIV-infected to the wells containing aliquots of the recombinant soluble T4/HIV mixture.

We adjusted each virus inoculum to a concentration of 250 $TCID_{50}$/ml. We preincubated 100 µl of the virus inoculum with 200 µl recombinant soluble T4 or 100 µl immunoglobulin prepared in triplicate serial 2-fold dilutions for 1 hour at 37°C prior to inoculation onto 1.5 - 2 x 10^6 H9 cells in 5 ml RPMI 1640 supplemented fetal calf serum (20%), HEPES (10mM), penicillin (250 U/ml), streptomycin (250 µg/ml) and L-glutamine (2mM). On days 5, 6, 7, 10 and 14, we examined each culture for characterisic cytopathic effects ("CPE"). Neutralization was defined as the inhibition of syncytia formation comared with controls.

The positive control used was HIV seropositive neutralizing serum, as described in D. D. Ho et al., "Human Immunodeficiency Virus Neutralizing Antibodies Recognize Several Conserved Domains On The Envelope Glycoproteins", J. Virol., 61, pp. 2024-28 (1987). The negative controls used were HIV seronegative serum only and buffer only.

Cytopathic Effect Assay (CPE)

In this assay, following conventional protocols for cytopathic effect assays [Klatzmann et al. (1984), supra and Wong-Staal and Gallo (1985), supra], we microscopically examined the H9 cells for evidence of cytopathic effects of HIV.

The CPE was scored on a four point scale from 1+ to 4+, with 4+ representing the highest degree of CPE.

By day 14, wells containing recombinant soluble T4 according to this invention (rsT4.2, derived from the pBG380 transfected CHO cell line BG380) at 10 µg/ml showed no evidence of CPE, while the negative control showed 1+ to 3+ CPE.

p24 Radioimmunoassay

We then tested soluble T4 as an inhibitor of viral replication in an HIV virus replication assay according to D. D. Ho et al., J. Virol., 61, pp. 2024-28 (1987) and J. Sodroski et al., Nature, 322, pp. 470-74 (1986). We carried out the assay essentially as described, except that the cultures were propagated in microtiter wells containing 200 µl. In this assay, we evaluated the ability of the soluble T4 polypeptides of this invention to block HIV replication, as measured by HIV p24 antigen production. We sampled supernatants twice weekly for HIV p24 antigen as described below.

We obtained an assay kit [HTLV-III p24 Radioimmunoassay System, Catalogue No. NEK-040, NEK-040A, Biotechnology Systems, New Research Products, Dupont] which contains affinity purified ^{125}I labelled HIV p24 antigen, a rabbit anti-p24 antibody and a second goat anti-rabbit antibody which is used to precipitate antigen-antibody complexes. We carried out the assay according to the protocol included with the kit. Accordingly, we mixed a sample to be assayed or one of a series of amounts of unlabelled p24 antigen with a fixed amount of ^{125}I labelled p24 and a fixed limited amount of rabbit anti-p24 antibody. We incubated the samples overnight at room temperature and then added a goat anti-rabbit immunoglobulin preparation for 5 minutes at 40°C. We centrifuged the samples in a microfuge and aspirated the supernatant fluid. Pelletted ^{125}I labelled p24 was quantitated for each sample by gamma counting and a standard curve for the ^{125}I p24 displaced by the known amounts of antigen added to standard tubes was constructed. We then calculated the ^{125}I labelled p24 displaced by the antigen present in the unknown samples by interpolation using the standard curve constructed from the known amounts of p24 antigen contained in the standard samples. The results are shown in the table below.

p24 ASSAY OF HIV REPLICATION INHIBITION

Day	rsT4.2 (μg/ml)	Patient Serum	Average CPM	% Bound/ Unbound
7	-	Negative	344	8.5
5	-	Positive	2,237	112.4
	0.5*	-	551	19.9
	5.0**	-	1,766	86.6
10	-	Negative	230	2.2
	-	Positive	2,459	124.6
10	0.5*	-	322	7.3
	5.0**	-	1,980	96.3
14	-	Negative	221	1.8
	-	Positive	2,284	115.0
	0.5*	-	246	3.1
15	5.0**	-	1,988	98.7

These results demonstrate that soluble T4 according to this invention at a concentration of 5 μg/ml completely inhibits virus replication as measured in this standard 14 day assay. These 20 results are also depicted in Figure 39 in graphic form. In Figure 39, values were calculated from a standard curve of p24 according to assay kit instructions.

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25 * This concentration was initially believed to be 1.0 μg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Absorbance at 280 nm is a commonly used first approximation of protein 30 concentration. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

35 ** This concentration was initially believed to be 10 μg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Absorbance at 280 nm is a commonly used first approximation of protein 40 concentration. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

We then carried out a p24 replication assay as described above, except that the soluble T4 was added to the infected cultures during refeeding at days 3, 7 and 10, in order to maintain a constant rsT4 concentration throughout the infection period. The results of this assay are shown in the table below.

INHIBITION OF HIV REPLICATION
WITH CONSTANT CONCENTRATION OF rsT4

rsT4.2 (μg/ml)	p24 (ng/ml)
0.008	770
0.031	970
0.125	85
0.5	0
5.0	0
0	1120
uninfected	0

These results demonstrate that when soluble T4 protein according to this invention was maintained at a constant concentration throughout the infection period, as little as 0.125 μg/ml of the protein substantially blocked replication of 250 $TCID_{50}$/ml of HIV-1.

Advantageously, soluble T4 protein according to this invention, at concentrations far exceeding those required to block viral replication, did not exert immunotoxic effects in vitro, as measured by three lymphocyte proliferation assays -- mixed lymphocyte response, phytohemagglutinin, and tetanus toxoid stimulated response.

Syncytia Inhibition Assay

To further assess the effect of soluble T4 on HIV env-T4 binding, we evaluated the effect of two preparations of our soluble T4 protein on the syncytiagenic properties of HIV in the co-cultivation assay. We carried out a C8166 cell fusion assay

as described in B. D. Walker et al., Proc. Natl. Acad. Sci. USA, 84, pp. 8120-24 (1984).

We incubated 1 x 10^9 H9 cells chronically infected with HTLV-IIIB for 1 hour at 37°C in 5% CO_2 with various concentrations of one of two preparations of rsT4.2 in 150 µl RPMI-1640 media supplemented with 20% fetal calf serum. We then added 3 x 10^4 C8166 cells in 50 µl media (a $T4^+$ transformed human umbilical cord blood lymphocyte line [Sodroski et al., supra], to a final volume of 0.2 ml in each well. Final well concentrations of soluble T4 were 0.5 µg/ml* and 5.0 µg/ml* for preparation #1 and 1.25 µg/ml* and 12.5 µg/ml* for preparation #2. We then counted total number of syncytia per well at 2 hours and 4 hours after adding the C8166 cells at 37°C in 5% CO_2. Parallel co-cultivations used buffer alone (negative control) or OKT4A at 25 µg/ml (positive control) as controls. We considered a positive result as a 50% reduction in syncytia compared to controls, at a time when at least 100 syncytia per 10^4 infected H9 cells were present in the control cultivations. The results of this assay are shown below and in Figure 40 (2 hour data).

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* These concentrations were initially believed to be, respectively, 1 µg/ml, 10 µg/ml, 2.5 µg/ml and 25 µg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

INHIBITION IN C8166 FUSION ASSAY

Preparation	[rsT4.2] (µg/ml)	% Inhibition* 2 Hrs	4 Hrs
buffer	0	0	0
rsT4.2	0.5**	30	42
rsT4.2	5.0**	54	47
rsT4.2	1.25**	16	21
rsT4.2	12.5**	77	55
OKT4A (25 µg/ml)	0	100	100

As demonstrated in this table and in Figure 40, soluble T4 according to this invention at 5.0 µg/ml and 12.5 µg/ml inhibited syncytia formation at 2 hours, as compared to buffer alone. By 4 hours after the addition of C8166 cells, soluble T4 at 12.5 µg/ml continued to inhibit greater than 50% syncytia formation, as compared to the negative control.

We also evaluated the effect of two preparations of our soluble T4 protein rsT4.7 on the syncytiagenic properties of HIV in a similar cocultivation assay. The results of this assay are shown below.

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* All assays were carried out in triplicate, and the number of syncytia counted per well was averaged to calculate % inhibition. The % inhibition represents the difference between the average number of syncytia in the negative control (without rsT4 or OKT4A) and the average number of syncytia counted when either rsT4 or OKT4A were present during the assay, divided by the average syncytia count for the negative control and multiplied by 100.

** These concentrations were initially believed to be, respectively, 1 µg/ml, 10 µg/ml, 2.5 µg/ml and 25 µg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Upon amino acid analysis of the protein however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

INHIBITION IN C8166 FUSION ASSAY

Assay date: day 1

Preparation	rsT4.7 (µg/ml)	Average Syncytia/50µl aliquot	% Inhibition at 2 Hrs
H9 cells (control)	0	0	N/A
C8166 cells (control)	0	0	N/A
HIV-infected H9 cells added to C8166 cells (control)	0	118	0
OKT4A (control)	0	0	100
Prep. 1 of rsT4.7	≅ 5.0*	43	63.6

* This concentration was initially believed to be 10 µg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

Assay date: day 13

Preparation	rsT4.7 (µg/ml)	Average Syncytia/50µl aliquot	% Inhibition at 2 Hrs
H9 cells (control)	0	0	N/A
C8166 cells (control)	0	1	N/A
HIV-infected H9 cells added to C8166 cells (control)	0	141	0
OKT4A (control)	0	0	100
Prep. 2 of rsT4.7	≅ 5.0*	27	80.9

* This concentration was initially believed to be 10 µg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

Assay date: day 14

Preparation	rsT4.7 (µg/ml)	Average Syncytia/50µl aliquot	% Inhibition at 2 Hrs
H9 cells (control)	0	0	N/A
C8166 cells (control)	0	0	N/A
HIV-infected H9 cells added C8166 cells (control)	0	128	0
OKT4A (control)	0	0	100
Prep. 1 of rsT4.7	≅ 5.0*	35	72.7
Prep. 2 of rsT4.7	≅ 5.0*	2	98.4

As demonstrated in these tables, soluble T4 protein rsT4.7 inhibited syncytia formation in HIV-infected H9 cells.

We also evaluated the effect of rsT4.113.1 and rsT4.111 on the syncytiagenic properties of HIV in a co-cultivation assay. We carried out a C8166 cell fusion assay as described in Walker et al., supra.

We incubated 1 x 10^4 H9 cells chronically infected with HTLV-IIIB for 1 hour at 37°C in 5% CO_2, with from 5 to 50 µg/ml rsT4.113.1 or rsT4.111 in 150 µl RPMI-1640 media supplemented with 20% fetal calf serum in 96-well microtiter plates. We

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* This concentration was initially believed to be 10 µg/ml, based upon our preliminary approximation that 1 unit of absorbance at 280 nm ("A_{280}"), was equivalent to 1 mg of rsT4.2. Upon amino acid analysis of the protein, however, we found that it had a higher extinction coefficient than originally approximated, with 1 A_{280} unit of rsT4.2 being equivalent to 0.5 mg of the protein.

then added 3 x 10^4 C8166 cells to the wells in 50 µl aliquots. The plates were incubated for 2 hours at 37°C in 5% CO_2 and, following this incubation, the number of syncytia per well were counted.

Syncytia were defined as cells containing a ballooning cytoplasm greater than three cell diameters. All samples were counted twice. Parallel co-cultivation used OKT4A alone or rsT4.3 alone at a concentration of 25 µg/ml (positive controls) or H9 cells alone or C8166 cells alone (negative controls). The results of this assay are shown below and in Figure 41.

INHIBITION IN C8166 FUSION ASSAY

Preparation	rsT4(µg/ml)	% Inhibition
H9 cells (control)	0	0
C8166 cells (control)	0	0
rsT4.113.1	1.25	35
rsT4.113.1	2.5	63
rsT4.113.1	4.25	63
rsT4.113.1	6.25	82
rsT4.113.1	12.5	96
rsT4.3	12.5	100
OKT4A (25 µg/ml)	0	100

As demonstrated in this table and in Figure 41, rsT4.113.1 exhibited a dose-dependent inhibition of HIV-induced syncytia formation. The molar specific inhibitory activity of rsT4.113.1 appeared to be reduced by an order of magnitude by comparison to anti-viral activity of longer forms of recombinant soluble T4. Thus, whereas rsT4.113.1 is effective toward neutralization of HIV-dependent cell fusion in vitro, its molar specific inhibitory

activity is decreased by a factor of 10. It is undetermined whether this decreased potency is due to incomplete renaturation of the E.coli-derived protein, the presence of three additional amino acids at the N-terminus of rsT4.113.1 (Met-Gln-Gly) lacking in rsT4.2 or rsT4.3 produced in mammalian cells, or the absence of additional structure in rsT4.113.1 required for high-affinity binding to HIV.

We also carried out a C8166 cell fusion assay with rsT4.111, as described for rsT4.113.1. The results of this assay are shown below.

INHIBITION IN C8166 FUSION ASSAY

Preparation	rsT4(µg/ml)	% Inhibition
H9 cell (control)	0	0
C8166 cells (control)	0	0
rsT4.111	1.25	0
rsT4.111	2.5	40
rsT4.111	4.25	20
rsT4.111	6.25	67
rsT4.111	12.5	100
rsT4.111	25.0	100
rsT4.3	12.5	100
rsT4.3	25.0	100
OKT4A (25 µg/ml)	0	100

As demonstrated in this table, rsT4.111 exhibited a dose-dependent inhibition of HIV-induced syncytia formation. At a concentration of 12.5 µg/ml and 25.0 µg/ml, complete inhibition of cell fusion was achieved.

Kinetics Of Intramuscular Injection Of Soluble T4

We examined the kinetics of the appearance of a recombinant soluble T4 protein according to this invention (specifically, rsT4.3 from the pBG381-transfected cell line BG381) in serum after intramuscular injection as follows.

We obtained two cynomolgus monkeys (Macaca fascicularis) who were free of infectious disease and in good health. Each monkey had been subjected to a 6 week quarantine period prior to administration of the soluble T4 protein. Throughout the administration period, each monkey was maintained on a conventional diet of monkey chow supplemented with fresh fruit. A catheter and a vascular access port were surgically placed in a femoral vein of each animal prior to treatment in order to facilitate blood collection.

Over a period of 28 days, each animal received recombinant soluble T4 protein twice daily by intramuscular injection to the large muscles of the thighs or buttocks. Injections were administered to each animal 8 hours apart and each injection contained a volume of 0.15 ml/kg (0.25 mg/kg) of rsT4.3 (from the pBG381-transformed cell line BG381), for a total dose of 0.5 mg/kg/day/monkey. Serum samples for clearance determination were collected on day 1 before the first treatment and at 1, 2, 4 and 8 hours after the first injection, as well as 1, 2, 4, 14 and 16 hours after the second injection on days 7, 14 and 28.

We found that intramuscularly injected soluble T4 reached the maximum level in serum between 1 and 2 hours after injection, with the level falling off slowly and reaching half-maximum value at approximately 6 hours post-injection. According to data obtained for intravenous administration (not shown), the level of rsT4.3 in serum should drop below that attained via intramuscular injection aproximately 2 hours after intravenous injection. Thus, while the maximum rsT4.3 level in serum after intramuscular injection does not reach that attainable via intravenous injection, it is slowly released into the blood stream, remaining detectable in serum for a

much longer time. This slow release mechanism associated with intramuscular routes of injection is advantageous because a higher level of soluble T4 protein is available over a longer period of time over a given concentration; thus remaining in a sustained level. Intramuscular administration of soluble T4 protein is particularly useful in treating early stage HIV-infected patients, to prevent the virus from disseminating, or in treating patients who have been exposed to the virus and who are not yet seropositive.

We determined serum levels of rsT4.3 using an ELISA assay. Throughout this assay, dilutions were made in blocking solution and, between each step, we washed the plates with PBS/0.05% Tween-20. More specifically, we coated wells of Immulon 2 plates with .01 OD (280 nm)/ml of OKT4 (IgG2b) in 0.05 M bicarbonate buffer to a volume of 50 µl/well and incubated the plates overnight at 4°C. We then blocked the plates with 5% bovine serum albumin in PBS, 200 µl/well, and incubated for 30 minutes at room temperature.

Subsequently, we added 50 µl of sample or standard to each well, incubating for 4 hours at room temperature. We then added 50 µl/well of OKT4A at 0.1 µg/ml and incubated overnight at 4°C. Using a Hyclone Kit (Hyclone) we then carried out the following steps. First, we added 1 drop of rabbit anti-mouse IgG2a to each well and incubated the plates for 1 hour at room temperature. We then added 100 µl of peroxidase-labeled anti-rabbit IgG, diluted 1:4000 with 5% BSA/PBS to each well, and incubated for 1 hour at room temperature.

We prepared a substrate reagent as follows. We diluted substrate reagent 1:10 in distilled water and added two O-phenyl-ethylene-diamine ("OPD") chromophore tablets per 10 ml of substrate. We let

the mixture dissolve thoroughly by mixing with a vortex. Alternatively, a TMB peroxidase substrate system (Kirkegaard & Perry Catalogue #50-76-00) may be used. Subsequently, we added 100 µl of the chromophore solution to each well, incubated for 10-15 minutes at room temperature and then stopped the color development with 100 µl of 1N H_2SO_4. We then measured OD at 490 nm, using an ELISA plate reader.

The results of the assay are demonstrated in the tables below.

Monkey #7-91

	rsT4 Level (ng/ml)			
Time(hr)	Day 1	Day 7	Day 14	Day 28
0	22.7*	96.5	158.0	19.8
1	278.8	199.6	360.7	238.3
2	281.8	366.8	306.4	441.1
4	214.9	246.6	363.9	393.2
5				290.4
8	72.3	105.0	199.4	
9**	246.2			
10	259.6			
12	136.0			
22	23.8			
24	13.4			

Monkey #7-92

	rsT4 Level (ng/ml)			
Time(hr)	Day 1	Day 7	Day 14	Day 28
0	6.7*	56.0	106.3	60.9
1	87.2	225.8	178.0	437.7
2	254.2	377.9	253.2	770.6
4	170.0	167.3	308.2	821.5
5				898.3
8	118.9	101.2	176.5	
9**	405.1			
10	523.5			
12	371.5			
22	48.4			
24	39.4			

* - background

** - second injection administered after the collection of the 8 hour sample.

Polyvalent Forms Of Recombinant Soluble T4

Receptors may be characterized by their affinity for specific ligands, such that, at equilibrium, the intrinsic affinity (K_a) between monovalent receptor and monovalent ligand can be defined as $[RL]/[R_f][L_f]$, where [RL] is the concentration of receptor (R) bound to ligand (L) and $[R_f]$ and $[L_f]$ are the concentrations of free receptor and ligand,

respectively [P. A. Underwood, in Advances In Virus Research, ed. K. Maramorosch et al., 34, pp. 283-309 (1988)].

For a polyvalent receptor (with a valency of n) binding to a polyvalent ligand (with a valency of m), a functional affinity can be defined as $n[R_b]/n[R_f]m[L_f]$, where $[R_b]$ is the concentration of bound receptor sites, and $n[R_f]$ and $m[L_f]$ are, respectively, the concentrations of free receptor and ligand binding sites. The effect of increasing the valence (the number of binding sites) is to enhance the stability of ligand-receptor complexes. The affinity of a polyvalent receptor for a polyvalent ligand will depend on three factors: the intrinsic association constant of each binding site, the valency (number of binding sites) and the topicological relationship between the receptor and ligand binding sites. Under some circumstances, polyvalent binding interactions will lead to higher functional affinity. The decreased dissociation rate of polyvalent ligands with polyvalent receptors results in an increased functional affinity [C. L. Hornick and F. Karush, Immunochemistry, 9, pp. 325-40 (1972); I. Otterness and F. Karush, "Principles Of Antibody Reactions", in Antibody As A Tool, ed. J. J. Marchalonais and G.W. Warr, pp. 97-137 (1982)].

The simplest case for receptor polyvalency increasing functional affinity is represented by a bivalent soluble receptor, such as an antibody molecule, which has two identical ligand binding sites, each capable of independently binding antigen with equal affinity. If the antigen is displayed polyvalently, for example, chemically coupled to a solid support such that the spacing between antigenic sites can be bridged by the antibody's two antigen binding arms, the functional affinity of the antibody for the antigen coupled to the solid support would be

greater than the intrinsic affinity of the antibody binding site for the monovalent antigen [D. Crothers and H. Metzger, Immunochemistry, 9, pp. 341-57 (1972)]. Because virus particles represent polyvalent antigens, the greater functional affinity of antibodies for polyvalent antigens is an important factor for antibody-directed virus neutralization.

The association of recombinant soluble T4 and the HIV major envelope glycoprotein gp120 is an example of monovalent receptor binding to monovalent ligand. The affinity of this interaction has been measured, and the association between T4 and gp120 has a dissociation constant $K_d = 4 \times 10^{-9}$ M [L. Lasky et al., Cell, 50, pp. 975-88 (1987)].

Using the antibody analogy, we believe that polyvalent rsT4 will demonstrate a greater affinity for HIV-infected cells displaying gp120 than monovalent rsT4 and the topicological relationship between gp120 on the virus particle or the infected cell surface, will determine the degree to which polyvalent rsT4 exhibits higher functional affinity than monovalent rsT4. One example of a polyvalent rsT4 is described below, with respect to the production of a recombinant bivalent rsT4 consisting of two tandem repeats of amino acids 3-178, followed by the C-terminal 199 amino acids of rsT4.3. According to this invention, a "polyvalent" receptor possesses two or more binding sites for a given ligand. Furthermore, the intrinsic affinity of each ligand binding site of a given polyvalent receptor need not be identical.

As shown in Figure 42, to construct bivalent rsT4, we digested pBG391 with NheI, which cleaves after the valine at position 178 in rsT4, and removed the NheI 5' overhang with mung bean nuclease. Next, we cleaved with BglII to remove the C-terminal half of the rsT4 coding sequence in pBG391. Finally, we

ligated a DraI-BglII fragment containing the coding sequence for rsT4 amino acids 3 (lysine) through 377 (isoleucine) to the cleaved pBG391 to create pBiv.1, a plasmid coding for a fusion protein with a tandem duplication of the N-terminal 176 amino acids of rsT4, followed by the C-terminal 199 amino acids of rsT4.3. The protein produced by this plasmid, therefore, contains two adjacent N-terminal gp120-binding or OKT4A-binding domains (defined by amino acid residues 3 through 111 of rsT4.111), followed by one OKT4-binding C-terminal domain (Figure 43).

pBiv.1 was transfected by electroporation into COS 7 cells to test expression of the bivalent rsT4 protein. Three days later, we tested the conditioned medium of the transfected cells for the presence of the rsT4 bivalent protein by immunoprecipitation, followed by Western blot analysis of the precipitated protein. Both OKT4A and OKT4 were used for immuno-precipitation to determine that the OKT4 epitope and at least one of the OKT4A epitopes had folded correctly. Both antibodies precipitated a protein of the predicted apparent molecular weight (60,000d) from the conditioned medium of the cells.

Bivalent rsT4 may be purified by immunoaffinity purification from an OKT4 column and the purified protein may then be used to perform quantitative competition assays with rsT4.3. We believe that the bivalent molecule would demonstrate equivalent competition against rsT4.3 for OKT4 binding, but significantly greater competition against monovalent rsT4 for OKT4A binding. The ability of bivalent recombinant soluble T4 to block syncytium formation may also be demonstrated in the C8166 fusion assay. We also believe that bivalent recombinant soluble T4 would block syncytium formation at significantly lower concentrations than monovalent rsT4; based upon the higher

functional affinity of bivalent recombinant soluble T4 for gp120.

According to alternate embodiments of this invention, other methods for producing polyvalent rsT4 may be employed. For example, polyvalent rsT4 may be produced by chemically coupling rsT4 to any clinically acceptable carrier molecule, a polymer selected from the group consisting of Ficoll, polyethylene glycol or dextran, using conventional coupling techniques. Alternatively, rsT4 may be chemically coupled to biotin, and the biotin-rsT4 conjugate then allowed to bind to avidin, resulting in tetravalent avidin/biotin/rsT4 molecules. And rsT4 may be covalently coupled to dinitrophenol (DNP) or trinitrophenol (TNP) and the resulting conjugate precipitated with anti-DNP or anti-TNP-Igm, to form decameric conjugates with a valency of 10 for rsT4 binding sites.

Alternatively, a recombinant chimeric antibody molecule with rsT4 sequences substituted for the variable domains of either or both of the immunoglobulin molecule heavy and light chains may be produced. Because recombinant soluble T4 possesses gp120 binding activity, the construction of a chimeric antibody having two soluble T4 domains and having unmodified constant region domains could serve as a mediator of targeted killing of HIV-infected cells that express gp120.

For example, chimeric rsT4/IgG_1 may be produced from two chimeric genes -- an rsT4/human kappa light chain chimera (rsT4/C_{kappa}) and an rsT4/human gamma 1 heavy chain chimera (rsT4/$C_{gamma-1}$). Both C_{kappa} and $C_{gamma-1}$ regions have been isolated from human recombinant DNA libraries, and each has been subcloned into animal cell selection vectors containing either the bacterial neo resistance or bacterial gpt markers

for selection in animal cell hosts against the antibiotic G418 or mycophenolic acid, respectively.

To construct rsT4/$C_{gamma-1}$ and rsT4/C_{kappa} chimeric genes, an rsT4 gene segment, including at least the secretory signal sequence and the N-terminal 110 amino acid residues of the mature rsT4 coding sequence and including a splice donor or portion thereof, is placed upstream of the gamma-1 and kappa constant domain exons. A suitable restriction enzyme may be used to cut within the intron downstream of the desired rsT4 coding sequence, thus providing a donor splice site. Subsequently, a suitable restriction enzyme is used to cut within the introns upstream of the kappa and gamma-1 coding regions. The rsT4 sequence is then joined to the kappa or gamma-1 constant region sequence, such that the rsT4 intron sequence is contiguous with the gamma-1 and kappa introns. In this way, an acceptor splice site is provided by the kappa or gamma-1 constant region intron. Alternatively, rsT4 chimeric genes may be constructed without the use of introns, by fusing a suitable rsT4 cDNA gene segment directly to the gamma-1 or kappa coding regions.

The rsT4/$C_{gamma-1}$ and rsT4/C_{kappa} vectors may then be cotransfected, for example, by electroporation into lymphoid or non-lymphoid host cells. Following transcription and translation of the two chimeric genes, the gene products may assemble into chimeric antibody molecules.

Expression of the chimeric gene products may be measured by an enzyme-linked immunoadsorbant assay (ELISA) that utilizes monoclonal anti-T4 antibody OKT4A, as described infra, or in gp120 competition assays and radioimmunoassays, as described infra. Activity of the rsT4/IgG_1 chimeras may be measured by incubating them with HIV-infected cells in the presence of human complement, followed by quantitating

subsequent complement-mediated lysis of these cells. Alternatively, activity may be measured in HIV replication and HIV syncytium assays as described infra.

In order to determine if bivalent rsT4 has a greater potency than monovalent rsT4, we mixed OKT4, at various concentrations, together with a constant concentration of rsT4, so that the molar ratio of OKT4:rsT4 varied between 0.2 and 4. After preincubating the mixture overnight at 4°C, we added aliquots to the HIV syncytium assay described infra. OKT4 has no observable effect in this assay when used alone. In addition, the concentration of recombinant soluble T4 chosen did not cause inhibition in this assay. Accordingly, we looked for indications that the OKT4/rsT4 mixture was more potent than rsT4 alone. We observed that at ratios of OKT4:rsT4 greater than 0.2, partial to complete inhibition of syncytium formation occurred. We believe that under conditions where two rsT4 molecules are bound to 1 OKT4 molecule, the greatest inhibitory effect should be found.

Thus, polyvalent, as well as monovalent forms of recombinant soluble T4 are useful in the compositions and methods of this invention.

Microorganisms and recombinant DNA molecules prepared by the processes of this invention are exemplified by cultures deposited in the In Vitro International, Inc. culture collection, in Linthicum, Maryland, on September 2, 1987, and identified as:

BG378: E.coli MC1061/pBG378
199-7: E.coli MC1061/p199-7
170-2: E.coli JA221/p170-2
EC100: E.coli JM83/pEC100
BG377: E.coli MC1061/pBG377
BG380: E.coli MC1061/pBG380
BG381: E.coli MC1061/pBG381

These cultures were assigned accession numbers IVI 10143-10149, respectively.

In addition, microorganisms and recombinant DNA molecules according to this invention are exemplified by cultures deposited in the In Vitro International, Inc. culture collection, in Linthicum, Maryland, on January 6, 1988, and identified as:

BG-391: E.coli MC1061/pBG391
BG-392: E.coli MC1061/pBG392
BG-393: E.coli MC1061/pBG393
BG-394: E.coli MC1061/pBG394
BG-396: E.coli MC1061/pBG396
203-5 : E.coli SG936/p203-5.

These cultures were assigned accession numbers IVI 10151-10156, respectively.

Microorganisms and recombinant DNA molecules according to this invention are also exemplified by cultures deposited in the In Vitro International, Inc. culture collection, in Linthicum, Maryland, on August 24, 1988 and identified as:

211-11: E.coli A89/pBG211-11
214-10: E.coli A89/pBG214-10
215-7 : E.coli A89/pBG215-7

These cultures were assigned accession numbers IVI 10183-10185 respectively.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic constructions can be altered to provide othe embodiments which utilize the processes and compositions of this invention. Therefore, it will be appreciated that the scope of this invention is to be defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

CLAIMS

We claim:

1. A DNA sequence selected from the group consisting of:

(a) the DNA inserts of p199-7, pBG377, pBG380, pBG381, p203-5, pBG391, pBG392, pBG393, pBG394, pBG395, pBG396, pBG397, p211-11, p214-10 and p215-7;

(b) DNA sequences which hybridize to one or more of the foregoing DNA inserts and which code on expression for a soluble T4-like polypeptide; and

(c) DNA sequences which code on expression for a soluble T4-like polypeptide coded for on expression by any of the foregoing DNA inserts and sequences.

2. The DNA sequence according to claim 1, wherein said DNA sequence (b) codes on expression for a soluble T4-like polypeptide which inhibits adhesion between $T4^+$ lymphocytes and infective agents which target $T4^+$ lymphocytes and which inhibits interaction between $T4^+$ lymphocytes and antigen presenting cells and targets of $T4^+$ lymphocyte mediated killing.

3. A recombinant DNA molecule comprising a DNA sequence selected from the group consisting of the DNA sequences of claim 1 or 2, said DNA sequence being operatively linked to an expression control sequence in said recombinant DNA molecule.

4. The recombinant DNA molecule according to claim 3, wherein said expression control sequence is selected from the group consisting of the early or late promoters of SV40 or adenovirus, the lac system, the trp system, the TAC system, the TRC

system, the major operator and promoter regions of phage λ, the control regions of fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid phosphatase, the polyhedron promoter of the baculovirus system and the promoters of the yeast α-mating factors.

5. A unicellular host transformed with a recombinant DNA molecule selected from the group consisting of the recombinant DNA molecules of claim 3 or 4.

6. The host according to claim 5, wherein said host is selected from the group consisting of strains of E.coli, Pseudomonas, Bacillus, Streptomyces, fungi, animal cells, plant cells, insect cells and human cells in tissue culture.

7. A polypeptide coded for on expression by a DNA sequence selected from the group consisting of the DNA sequences of claim 1 or 2, said polypeptide being essentially free of other proteins of human origin.

8. The polypeptide according to claim 7, wherein said polypeptide is selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{362} of Figure 3, a polypeptide of the formula AA_{1-362} of Figure 3, a polypeptide of the formula Met-AA_{1-362} of Figure 3, a polypeptide of the formula AA_{1-374} of Figure 3, a polypeptide of the formula Met-AA_{1-374} of Figure 3, a polypeptide of the formula AA_{1-377} of Figure 3, a polypeptide of the formula Met-AA_{1-377} of Figure 3, a polypeptide of the formula AA_{-23}-AA_{374} of Figure 3, a polypeptide of the formula AA_{-23}-AA_{377} of Figure 3.

9. The polypeptide according to claim 7, wherein said polypeptide is selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{182} of Figure 16, a polypeptide of the formula AA_1-AA_{182} of Figure 16, a polypeptide of the formula Met-AA_{1-182} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_1-AA_{182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula Met-AA_{1-182} of Figure 16, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_{-23}-AA_{113} of Figure 16, a polypeptide of the formula AA_1-AA_{113} of Figure 16, a polypeptide of the formula Met-AA_{1-113} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{111} of Figure 16, a polypeptide of the formula AA_1-AA_{111} of Figure 16, a polypeptide of the formula Met-AA_{1-111} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{131} of Figure 16, a polypeptide of the formula AA_1-AA_{131} of Figure 16, a polypeptide of the formula Met-AA_{1-131} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{145} of Figure 16, a polypeptide of the formula AA_1-AA_{145} of Figure 16, a polypeptide of the formula Met-AA_{1-145} of Figure 16, a polypeptide of the formula AA_{-23}-AA_{166} of Figure 16, a polypeptide of the formula AA_1-AA_{166} of Figure 16, a polypeptide of the formula Met-AA_{1-166} of Figure 16, or portions thereof.

10. The polypeptide according to claim 7, wherein said polypeptide is selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{362} of mature T4 protein, a polypeptide of the formula AA_{1-362} of mature T4 protein, a polypeptide of the formula Met-AA_{1-362} of mature T4 protein, a polypep-

tide of the formula AA_{1-374} of mature T4 protein, a polypeptide of the formula Met-AA_{1-374} of mature T4 protein, a polypeptide of the formula AA_{1-377} of mature T4 protein, a polypeptide of the formula Met-AA_{1-377} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{374} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{377} of mature T4 protein, or portions thereof.

11. The polypeptide according to claim 7, wherein said polypeptide is selected from the group consisting of a polypeptide of the formula AA_{-23}-AA_{182} of mature T4 protein, a polypeptide of the formula AA_1-AA_{182} of mature T4 protein, a polypeptide of the formula Met-AA_{1-182} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_1-AA_{182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula Met-AA_{1-182} of mature T4 protein, followed by the amino acids asparagine-leucine-glutamine-histidine-serine-leucine, a polypeptide of the formula AA_{-23}-AA_{113} of mature T4 protein, a polypeptide of the formula AA_1-AA_{113} of mature T4 protein, a polypeptide of the formula Met-AA_{1-113} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{111} of mature T4 protein, a polypeptide of the formula AA_1-AA_{111} of mature T4 protein, a polypeptide of the formula Met-AA_{1-111} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{131} of mature T4 protein, a polypeptide of the formula AA_1-AA_{131} of mature T4 protein, a polypeptide of the formula Met-AA_{1-131} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{145} of mature T4 protein, a polypeptide of the formula AA_1-AA_{145} of mature T4 protein, a polypeptide of the

formula Met-AA_{1-145} of mature T4 protein, a polypeptide of the formula AA_{-23}-AA_{166} of mature T4 protein, a polypeptide of the formula AA_{1}-AA_{166} of mature T4 protein, a polypeptide of the formula Met-AA_{1-166} of mature T4 protein, or portions thereof.

12. A method for producing a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11 comprising the step of culturing a unicellular host transformed with a recombinant DNA molecule selected from the group consisting of the recombinant DNA molecules of claim 3 or 4.

13. A pharmaceutical composition comprising an immunotherapeutic or immunosuppressive effective amount of a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11 and a pharmaceutically acceptable carrier.

14. A method for treating patients comprising the step of treating them in a pharmaceutically acceptable manner with a composition selected from the group consisting of the composition of claim 13.

15. The method according to claim 14, wherein the patient is treated by intramuscular injection of the composition.

16. A diagnostic composition for detecting or for monitoring the course of HIV infection comprising a diagnostic effective amount of a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11.

17. A method for detecting or for monitoring the course of HIV infection comprising the

step of employing as a diagnostic a composition selected from the group consisting of the compositions of claim 16.

18. A means for detecting or for monitoring the course of HIV infection comprising a composition selected from the group consisting of the compositions of claim 16.

19. A pharmaceutical composition comprising an immunotherapeutic or immunosuppressive effective amount of antibody to a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11 and a pharmaceutically acceptable carrier.

20. A method for treating patients comprising the step of treating them in a pharmaceutically acceptable manner with a composition according to claim 19.

21. The use of a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11 to purify HIV virus.

22. The use according to claim 20, wherein the HIV virus is purified from a biological sample.

23. A method for purifying HIV virus from a sample comprising the step of exposing the sample to a polypeptide selected from the group consisting of the polypeptides of any one of claims 7 to 11.

24. The method according to claim 22, wherein the sample is a biological sample.

25. A DNA sequence comprising the DNA insert of p170-2, said sequence coding on expression for a T4-like polypeptide.

26. A recombinant DNA molecule comprising a DNA sequence selected from the group consisting of the DNA sequence of claim 25, said DNA sequence being operatively linked to an expression control sequence in said recombinant DNA molecule.

27. A unicellular host transformed with a recombinant DNA molecule according to claim 26.

28. A polypeptide coded for on expression by a DNA sequence of claim 25, said polypeptide being essentially free of other proteins of human origin.

29. A pharmaceutical composition comprising an immunotherapeutic or immunosuppressive amount of a soluble protein receptor and a pharmaceutically acceptable carrier.

30. A method for treating patients comprising the step of treating them in a pharmaceutically acceptable manner with a pharmaceutical composition of claim 29.

31. A diagnostic composition for detecting or for monitoring the course of viral infection comprising a diagnostic effective amount of a soluble protein receptor.

32. A method for detecting or for monitoring the course of a viral infection comprising the step of employing as a diagnostic a diagnostic effective amount of a soluble protein receptor.

33. A means for detecting or for monitoring the course of a viral infection comprising a soluble protein receptor.

34. A DNA sequence selected from the group consisting of:

(a) the DNA insert of pBiv.1;

(b) DNA sequences which hybridize to the DNA insert of pBiv.1 and which code on expression for a polyvalent soluble T4-like polypeptide; and

(c) DNA sequences which code on expression for a polyvalent soluble T4-like polypeptide coded for by the DNA insert of pBiv.1.

35. A recombinant DNA molecule comprising a DNA sequence selected from the group consisting of the DNA sequences of claim 34, said DNA sequence being operatively linked to an expression control sequence in said recombinant DNA molecule.

36. A unicellular host transformed with a recombinant DNA molecule according to claim 35.

37. A polypeptide coded for on expression by a DNA sequence selected from the group consisting of the DNA sequences according to claim 34, said polypeptide being essentially free of other proteins of human origin.

38. The polypeptide according to claim 7, wherein said polypeptide is polyvalent.

39. A method for producing a polyvalent polypeptide comprising the steps of:

(a) culturing a unicellular host transformed with a recombinant DNA molecule according to claim 3 or 4 to produce a polypeptide; and

(b) coupling said polypeptide to a carrier to form a polyvalent polypeptide.

40. A DNA sequence comprising:

(a) a first portion comprising a DNA sequence coding for the constant region of an immunoglobulin light chain; and

(b) a second portion comprising a DNA sequence according to claim 1 or 2, or portions thereof, said second portion being joined upstream of said first portion.

41. A DNA sequence comprising:

(a) a first portion comprising a DNA sequence coding for the constant region of an immunoglobulin heavy chain; and

(b) a second portion comprising a DNA sequence according to claim 1 or 2, or portions thereof, said second portion being joined upstream of said first portion.

42. An expression vector comprising the DNA sequence according to claim 40.

43. An expression vector comprising the DNA sequence according to claim 41.

44. An expression vector comprising the DNA sequence according to claim 40 and the DNA sequence according to claim 41.

45. A method for producing a chimeric rsT4/IgG_1 comprising the step of co-transfecting a host cell with the expression vector according to claim 42 and the expression vector according to claim 43.

46. A method for producing a chimeric rsT4/IgG_1 comprising the step of transfecting a host cell with the expression vector according to claim 44.

47. A chimeric rsT4/IgG_1 produced by the method according to claim 45 or 46.

48. A pharmaceutical composition comprising an immunotherapeutic or immunosuppressive effective amount of a polypeptide according to claim 37 or 38.

49. A method for treating patients comprising the step of treating them in a pharmaceutically acceptable manner with a composition according to claim 48.

50. A diagnostic composition for detecting or for monitoring the course of HIV infection comprising a diagnostic effective amount of a polypeptide according to claim 37 or 38.

51. A pharmaceutical composition comprising an immunotherapeutic or immunosuppressive effective amount of a chimeric rsT4/IgG_1 according to claim 47.

52. A method for treating patients comprising the step of treating them in a pharmaceutically acceptable manner with a composition according to claim 51.

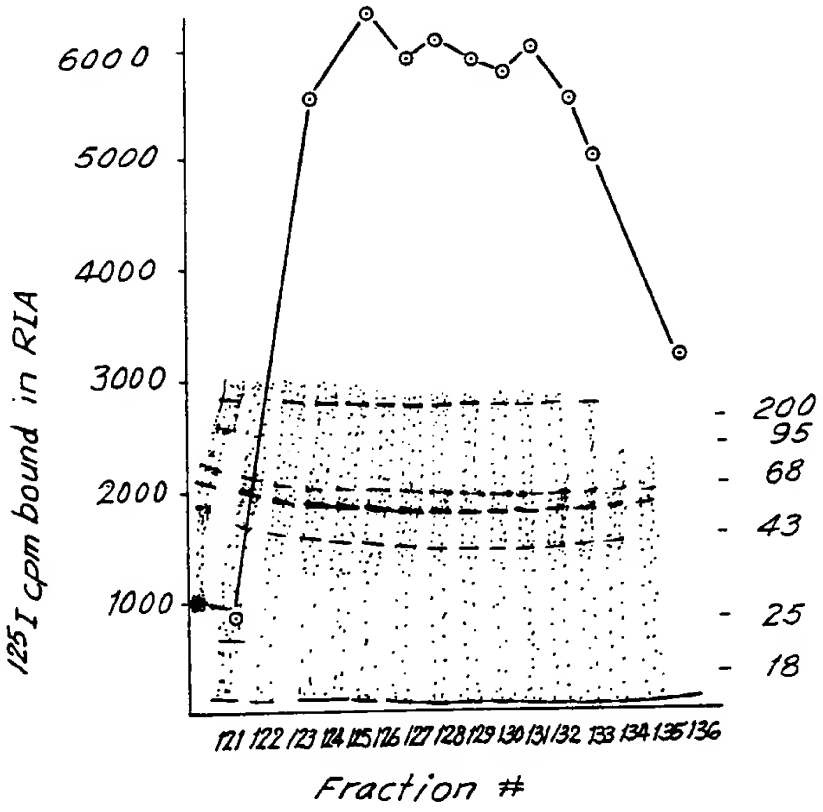
6000
5000
4000
3000
2000
1000
^{125}I cpm bound in RIA
200
95
68
43
25
18
121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136
Fraction #

FIG. 1

FIG. 2

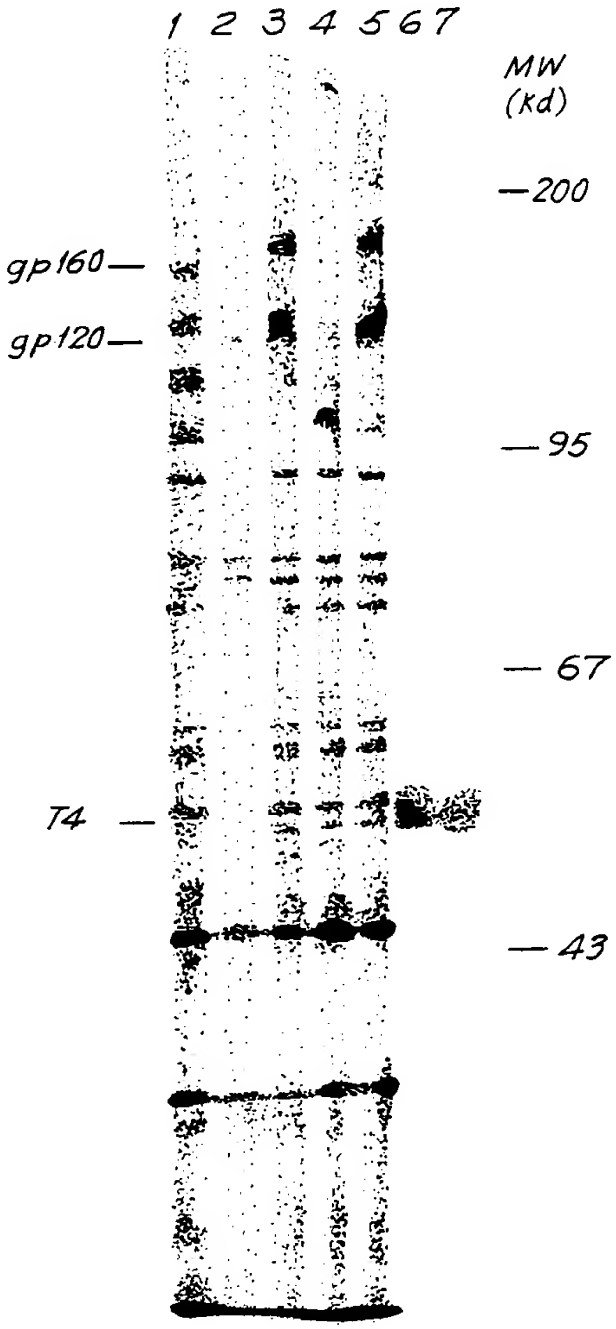

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FIG. 3

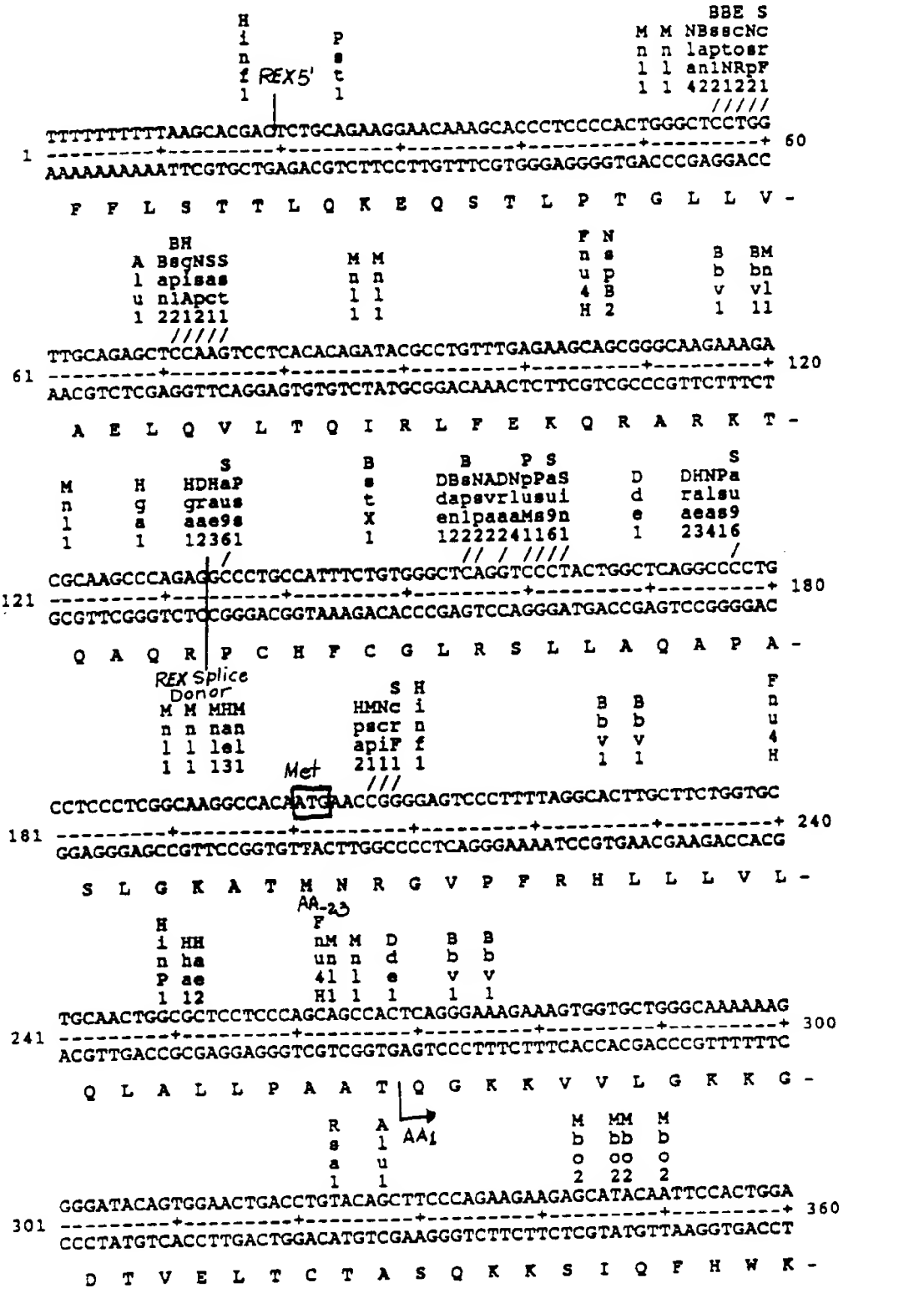
REX5'
REX Splice Donor
Met
AA-23
AA1
TTTTTTTTTTAAGCACGACTCTGCAGAAGGAACAAAGCACCCTCCCCACTGGGCTCCTGG
AAAAAAAAAATTCGTGCTGAGACGTCTTCCTTGTTTCGTGGGAGGGGTGACCCGAGGACC
F F L S T T L Q K E Q S T L P T G L L V -
TTGCAGAGCTCCAAGTCCTCACACAGATACGCCTGTTTGAGAAGCAGCGGGCAAGAAAGA
AACGTCTCGAGGTTCAGGAGTGTGTCTATGCGGACAAACTCTTCGTCGCCCGTTCTTTCT
A E L Q V L T Q I R L F E K Q R A R K T -
CGCAAGCCCAGACGCCCTGCCATTTCTGTGGGCTCAGGTCCCTACTGGCTCAGGCCCCTG
GCGTTCGGGTCTGCGGGACGGTAAAGACACCCGAGTCCAGGGATGACCGAGTCCGGGGAC
Q A Q R P C H F C G L R S L L A Q A P A -
CCTCCCTCGGCAAGGCCACAATGAACCGGGGAGTCCCTTTTAGGCACTTGCTTCTGGTGC
GGAGGGAGCCGTTCCGGTGTTACTTGGCCCCTCAGGGAAAATCCGTGAACGAAGACCACG
S L G K A T M N R G V P F R H L L L V L -
TGCAACTGGCGCTCCTCCCAGCAGCCACTCAGGGAAAGAAAGTGGTGCTGGGCAAAAAAG
ACGTTGACCGCGAGGAGGGTCGTCGGTGAGTCCCTTTCTTTCACCACGACCCGTTTTTTC
Q L A L L P A A T Q G K K V V L G K K G -
GGGATACAGTGGAACTGACCTGTACAGCTTCCCAGAAGAAGAGCATACAATTCCACTGGA
CCCTATGTCACCTTGACTGGACATGTCGAAGGGTCTTCTTCTCGTATGTTAAGGTGACCT
D T V E L T C T A S Q K K S I Q F H W K -

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FIG. 3(cont'd)

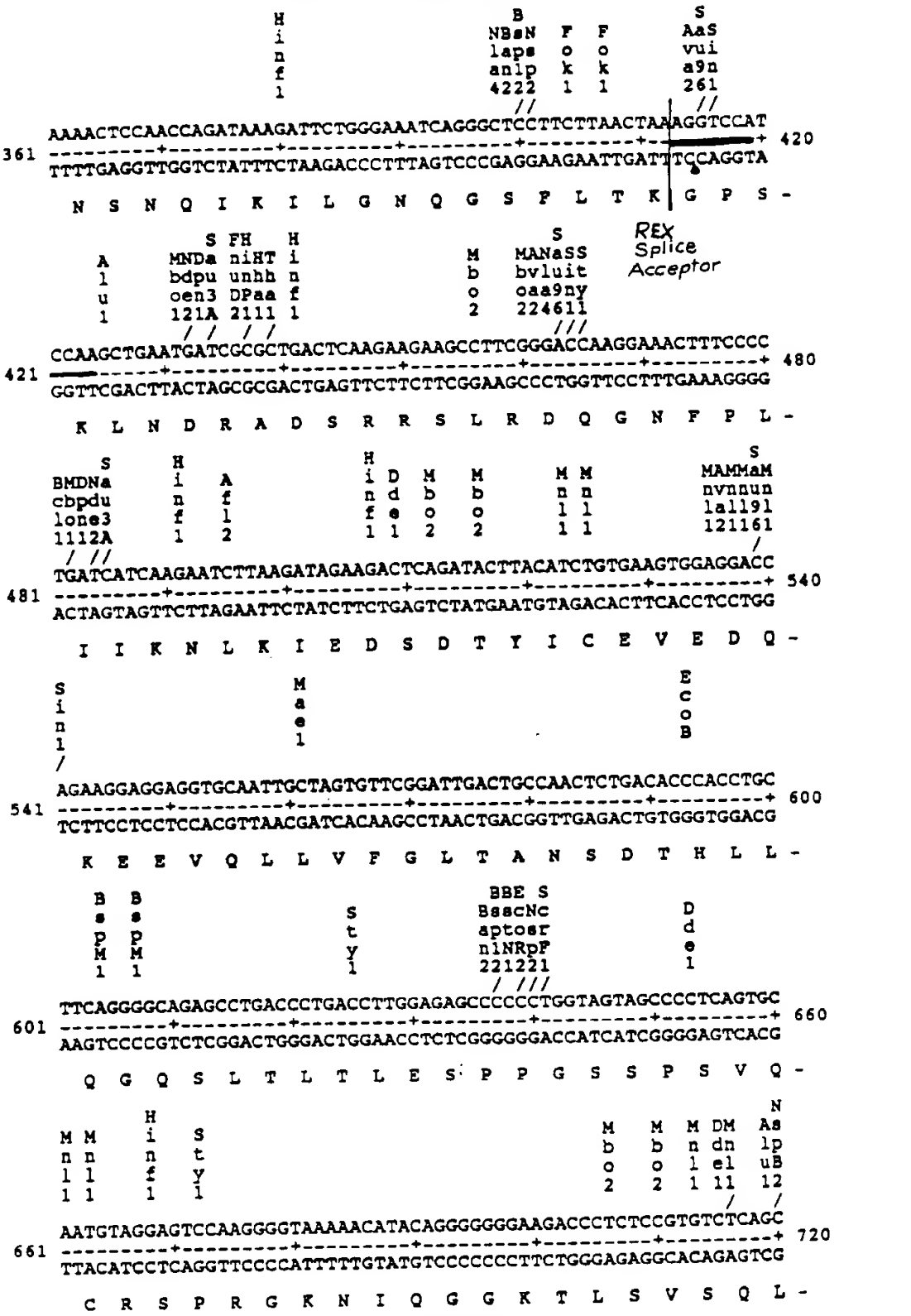

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FIG. 3(cont'd)

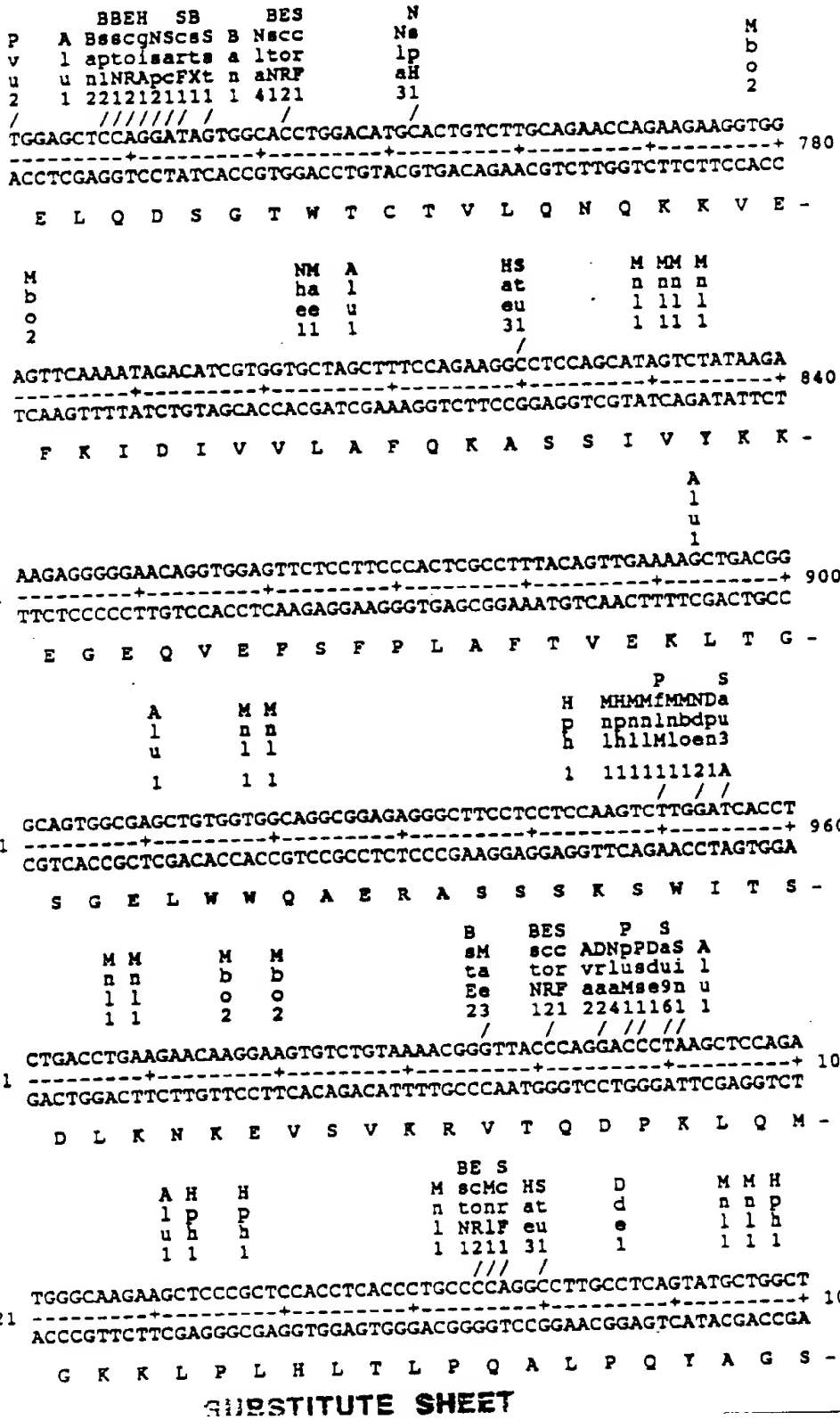

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FIG. 3(cont'd)

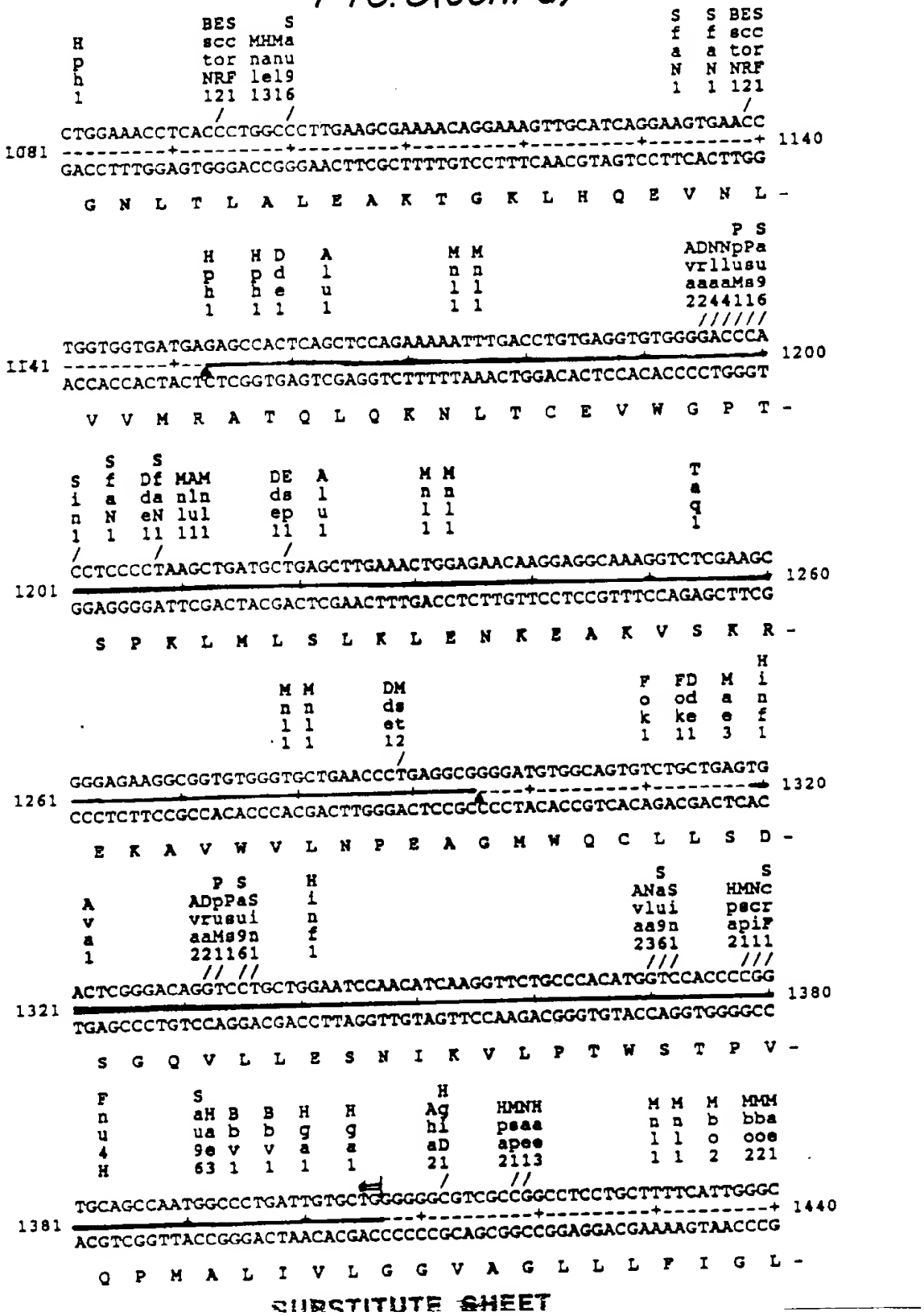

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FIG. 3(cont'd)

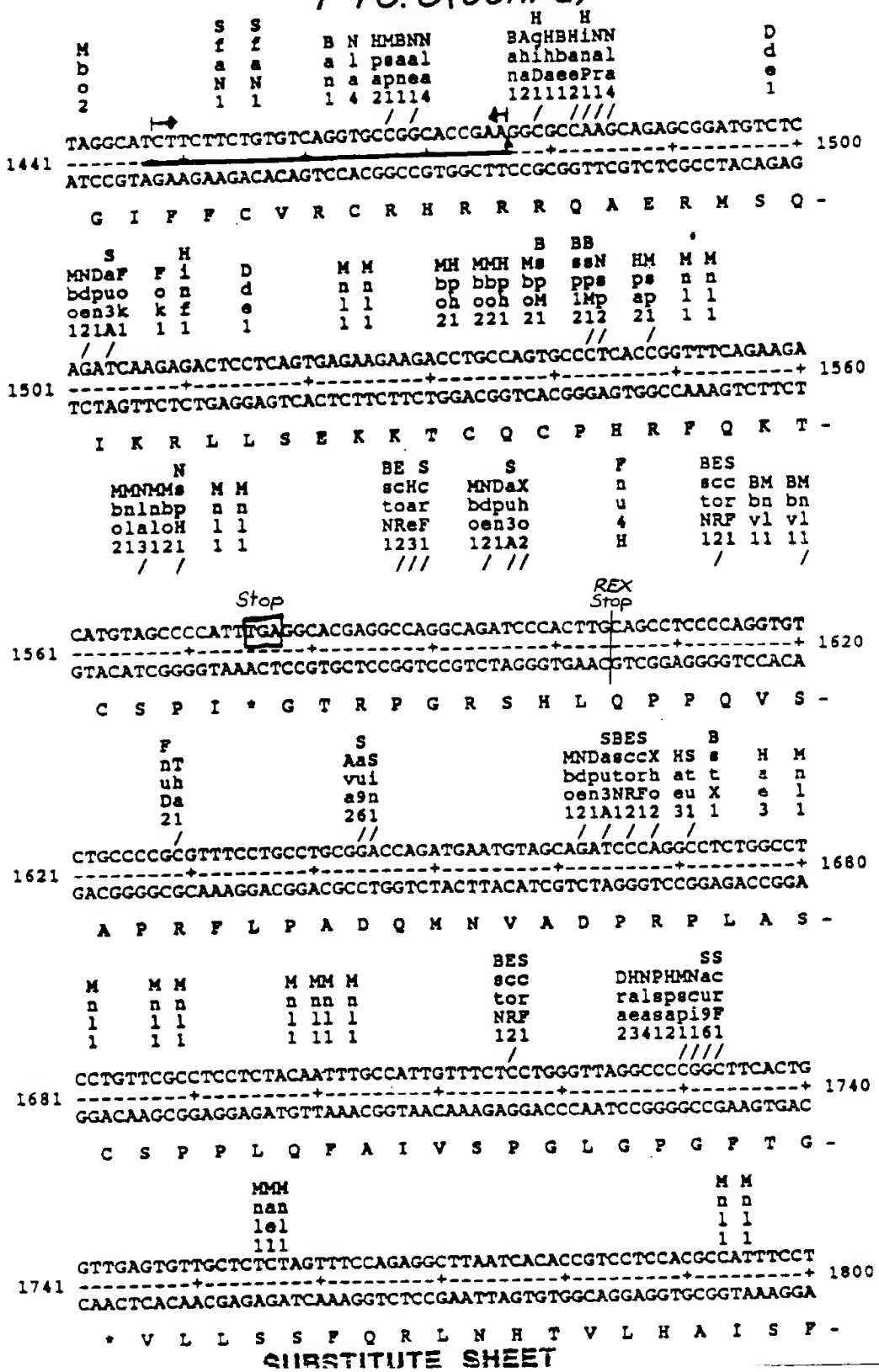

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FIG. 3(cont'd)

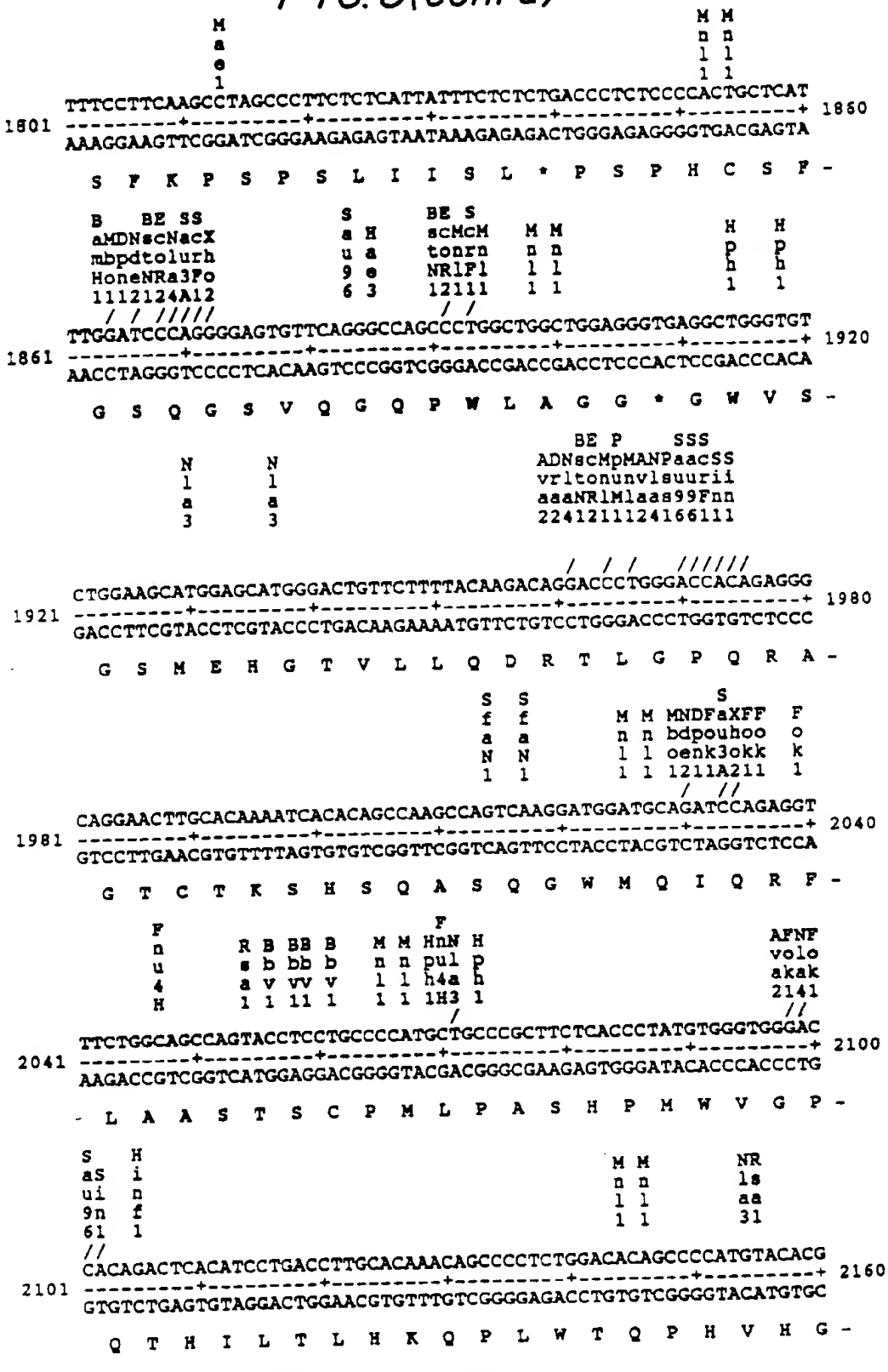

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FIG. 3(cont'd)

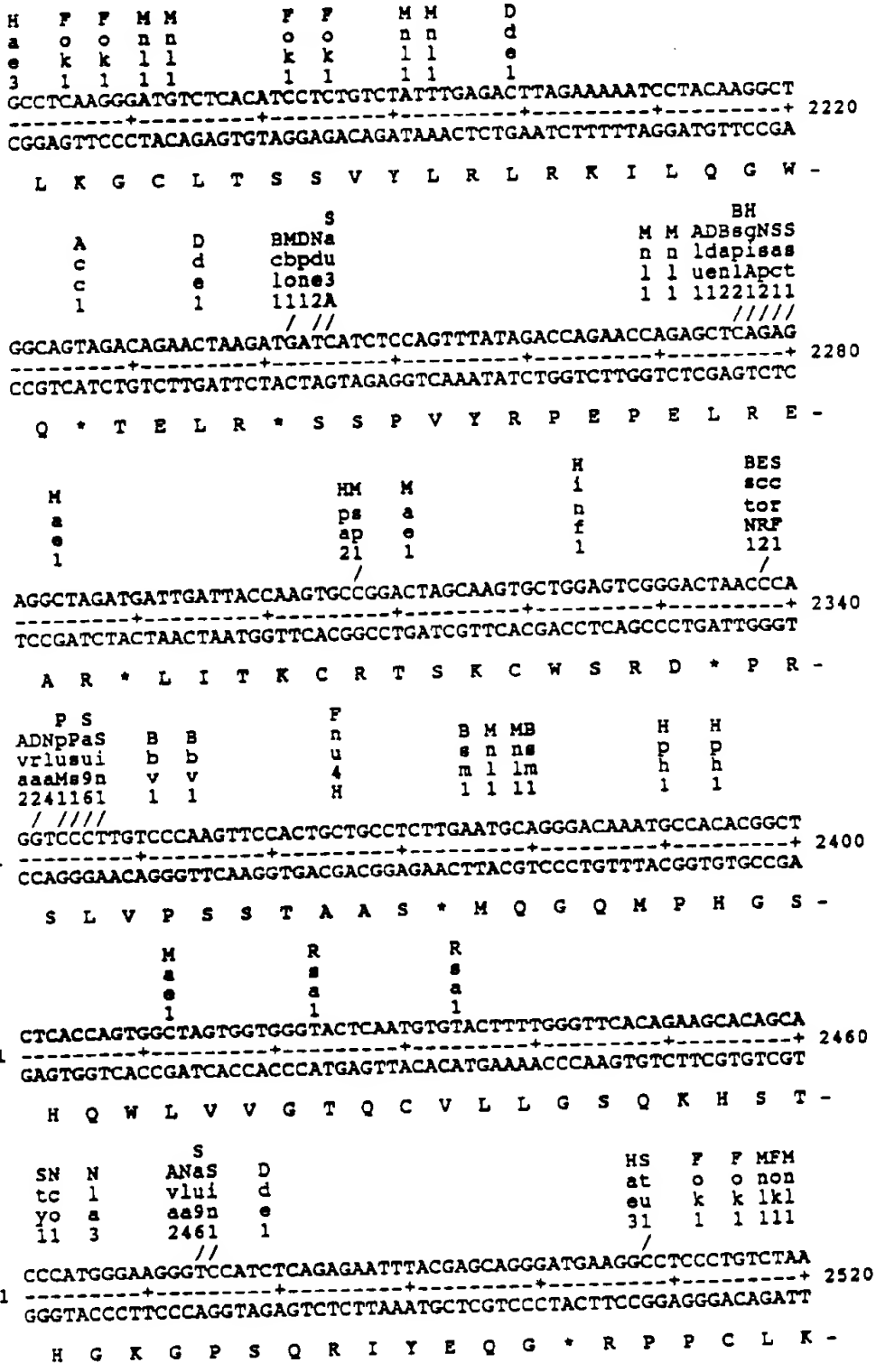

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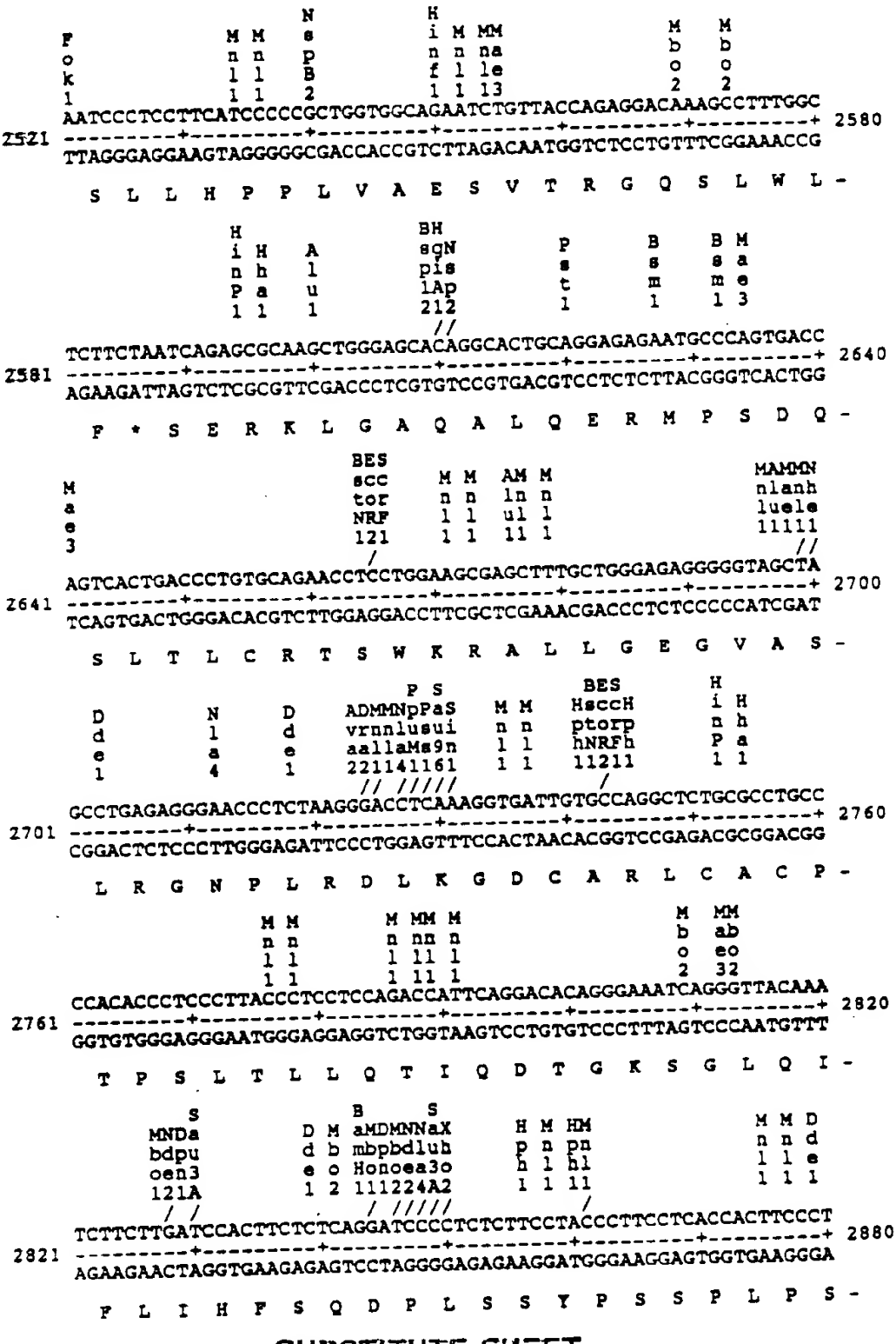
FIG. 3(cont'd)



SUBSTITUTE SHEET

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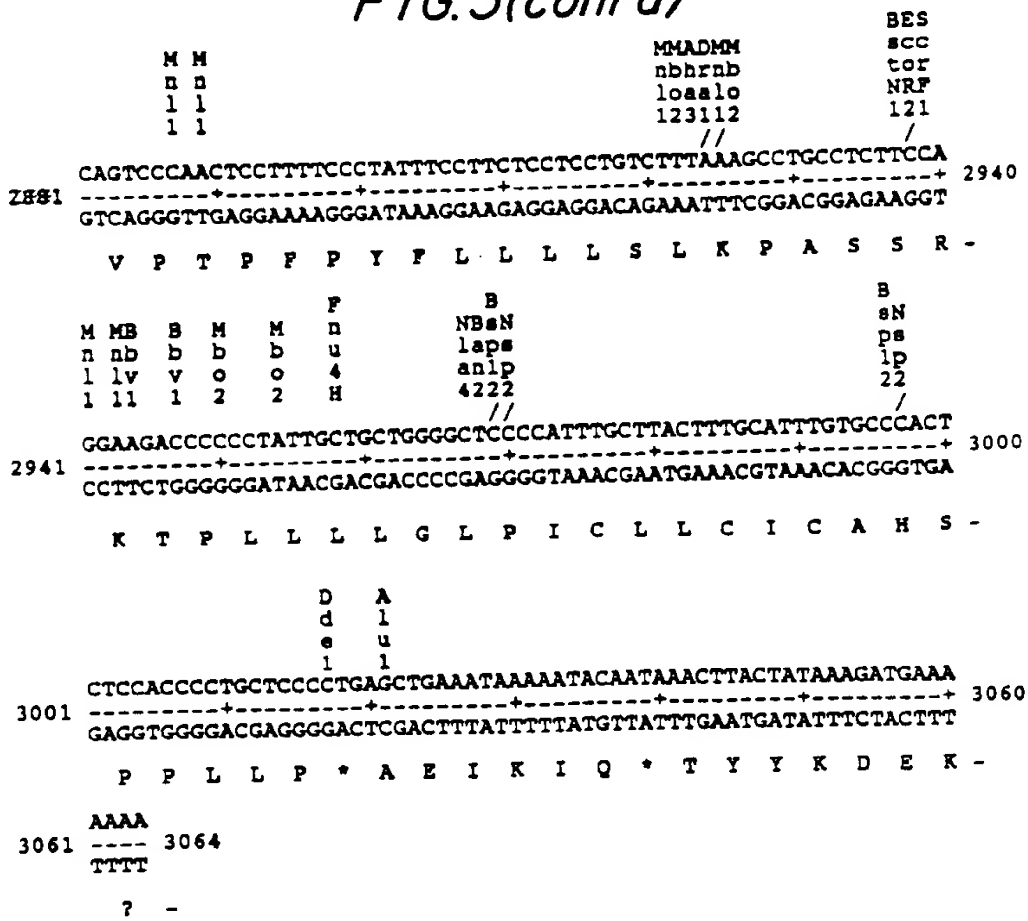
FIG. 3(cont'd)



Enzymes that do cut:

Acc1	Aha2	Aha3	Af12	Alu1	Ava1	Ava2	BamH1
Ban1	Ban2	Bbe1	Bbv1	Bc11	Bsm1	Bsp12	BspM1
BstE2	BstN1	BstX1	Dde1	Dpn1	Dra1	Dra2	EcoB
EcoR2	Esp1	FnuD2	Fnu4H	Fok1	Hae2	Hae3	Hga1
HgiA1	HgiD1	Hha1	Hinf1	HinP1	Hpa2	Hph1	Mae1
Mae3	Mbo1	Mbo2	Mn11	Msp1	Mst2	Nae1	Nar1
Nci1	Nco1	Nde2	Nhe1	N1a3	N1a4	Nsp2	NspB2
NspH1	Pf1M1	PpuM1	Pss1	Pst1	Pvu2	Rsa1	Sac1
Sau3A	Sau96	ScrF1	SfaN1	Sin1	Sst1	Stu1	Sty1
Taq1	Tha1	Xho2					

Enzymes that do not cut:

Aat2	Apa1	ApaL1	Asp70	Asp71	Asu2	Avr2	Bal1
Bgl1	Bgl2	BspM2	BssH2	Cfr1	Cla1	Dra3	Eag1
EcoK	EcoK	EcoR1	EcoRV	Fsp1	Hinc2	Hind3	Hpa1
Kpn1	Mae2	Mlu1	Mst1	Nde1	Not1	Nru1	Nsi1
PaeR7	Pvu1	Rsr2	Sac2	Sal1	Sca1	Sfi1	Sma1
SnaB1	Spe1	Sph1	Ssp1	Sst2	Tth1	Xba1	Xho1
Xma1	Xma3	Xmn1	Xor2				

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FIG. 4

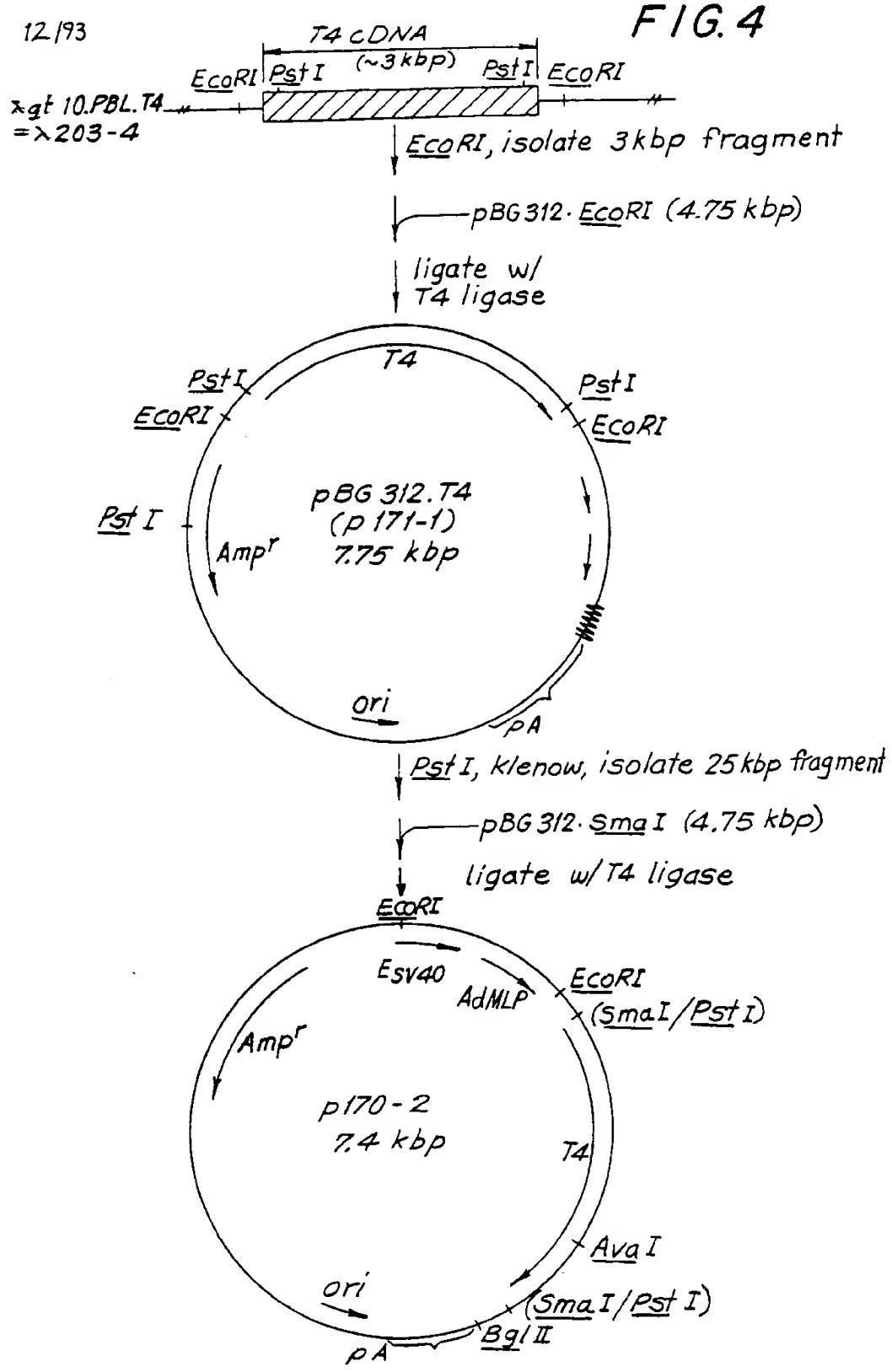
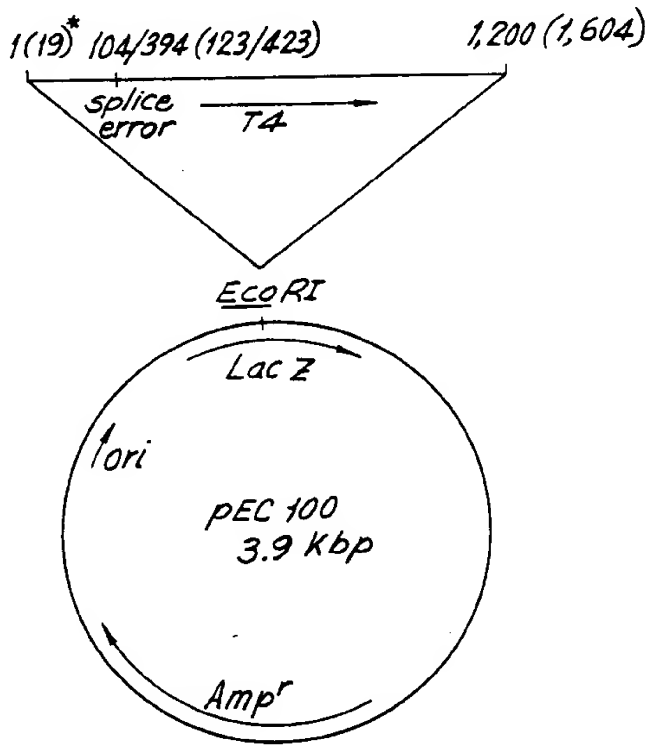

T4 cDNA
(~3 kbp)
EcoRI
PstI
PstI
EcoRI
λgt 10.PBL.T4
=λ203-4
EcoRI, isolate 3 kbp fragment
pBG312·EcoRI (4.75 kbp)
ligate w/
T4 ligase
T4
PstI
EcoRI
PstI
EcoRI
pBG 312.T4
(p 171-1)
7.75 kbp
PstI
Ampr
ori
pA
PstI, klenow, isolate 25 kbp fragment
pBG 312·SmaI (4.75 kbp)
ligate w/T4 ligase
EcoRI
Esv40
AdMLP
EcoRI
(SmaI/PstI)
Ampr
p170-2
7.4 kbp
T4
AvaI
ori
(SmaI/PstI)
BglII
pA

FIG. 5



*numbers in parentheses refer to PBL T4 cDNA coordinates

FIG. 6

AMINO ACID SEQUENCE COMPARISON AT POSITIONS 3, 6 AND 231 OF T4

Position No.	Maddon et al.	PBL Clone	Rex Clone	Genomic	Mouse	Sheep
3	N AAC	K AAG	-	-	K AAG	K -
64	W TGG	R CGG	W TGG	W TGG	W TGG	-
231	F TTT	S TCT	F TTT	-	F TTC	-

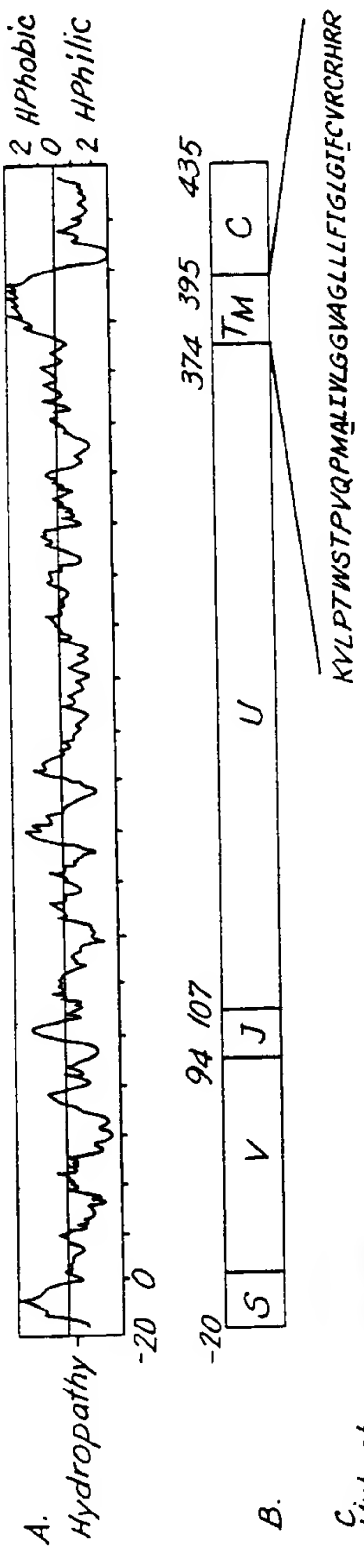

A.
Hydropathy
2 HPhobic
0
2 HPhilic
-20 0
B.
-20
S
V
94 107
J
U
374 395
TM
C
435
KVLPTWSTPVQPMALIVLGGVAGLLLFTGLGIFFCVRCRHRR
C.
Mutant
rsT4.1
rsT4.2
rsT4.3
M
-23
K COOH
362
M
374
COOH
I
377
D.
Met Perfect
(rsT4.2)
M 8
M
374
COOH

FIG. 7A

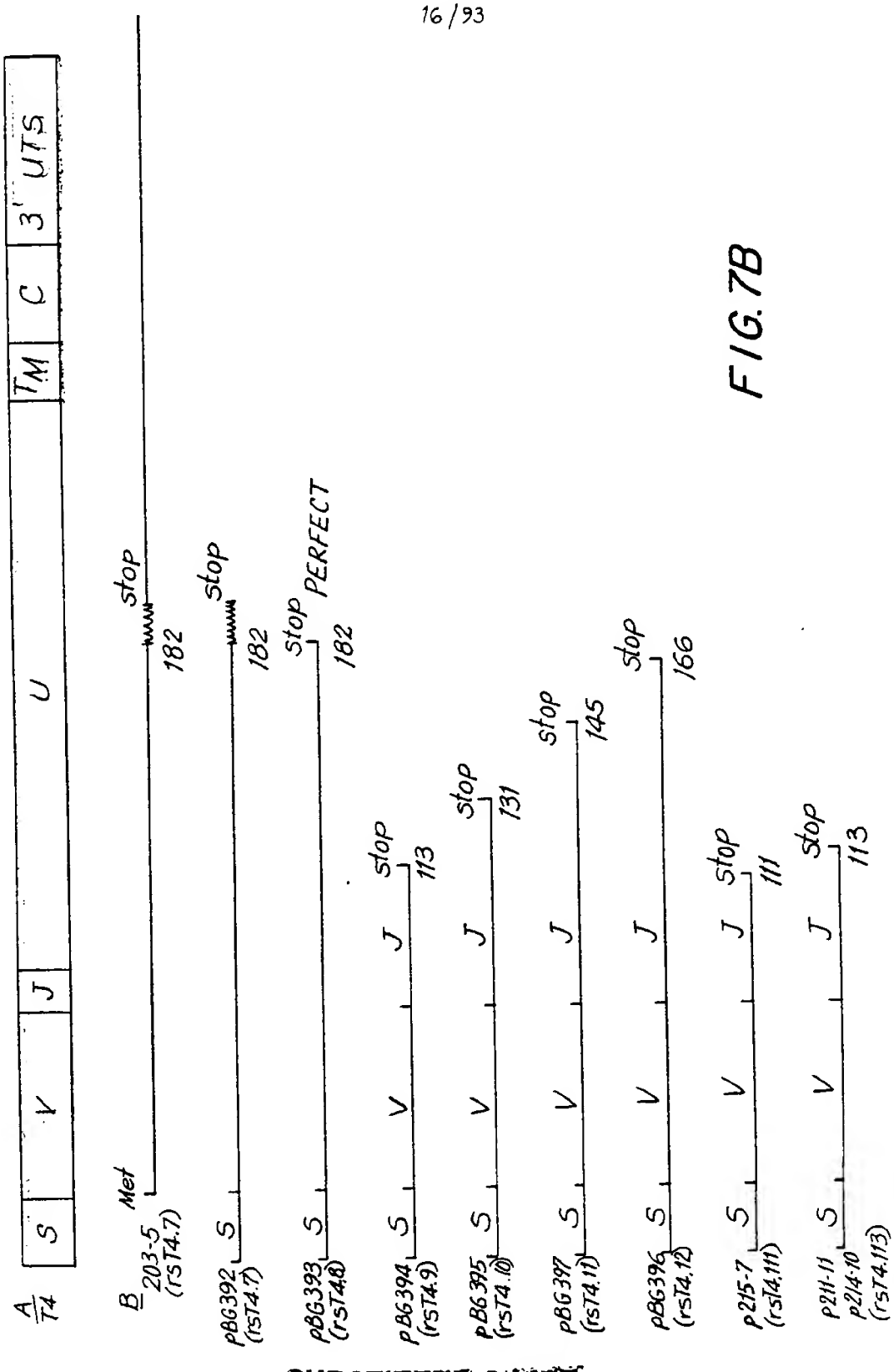
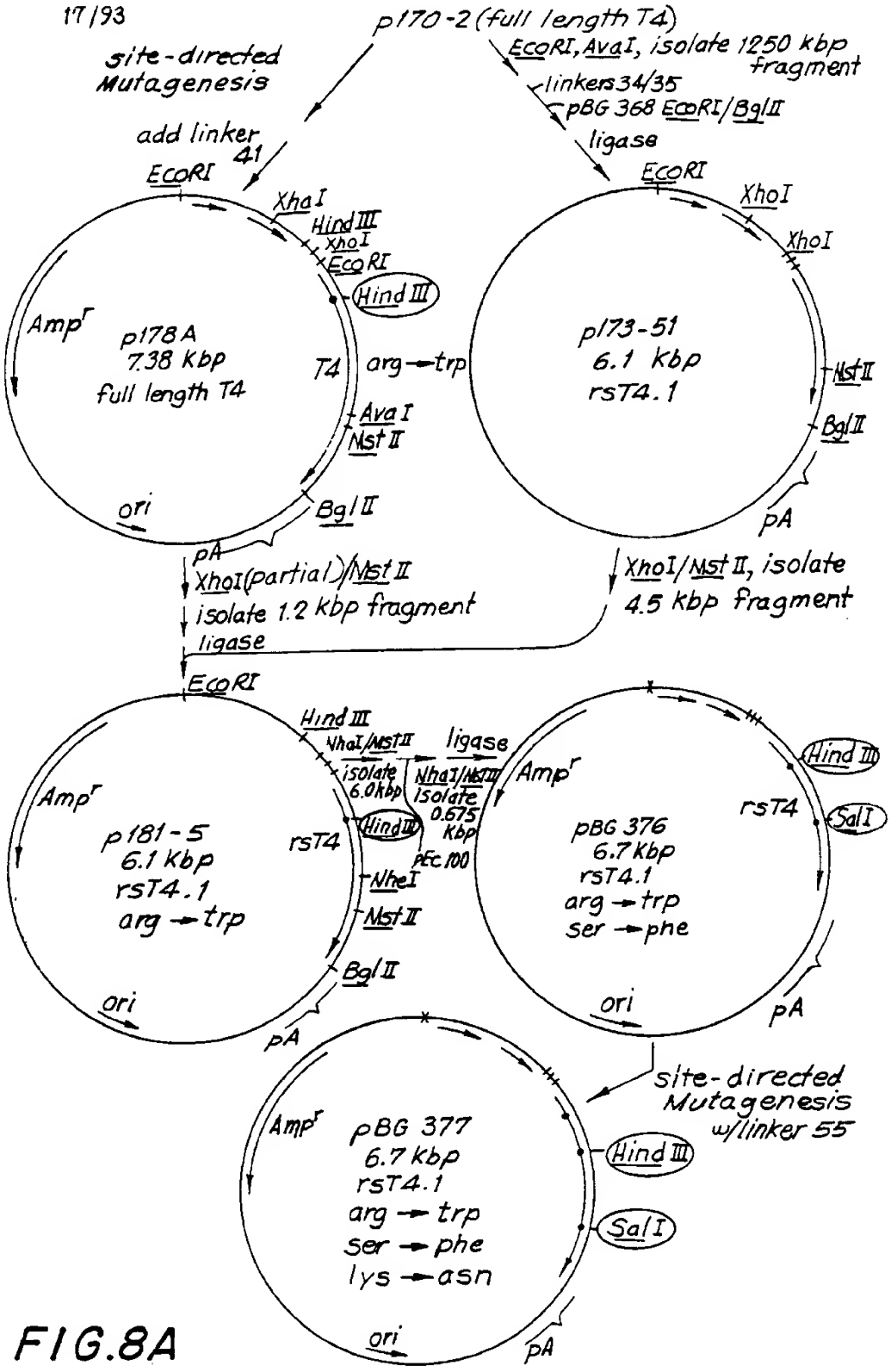

A
T4
S
V
J
U
TM
C
3' UTS
B
203-5
(rsT4.7)
Met
stop
182
pBG392
(rsT4.7)
S
stop
182
pBG393
(rsT48)
S
stop
PERFECT
182
pBG394
(rsT4.9)
S
V
J
stop
113
pBG395
(rsT4.10)
S
V
J
stop
131
pBG397
(rsT4.11)
S
V
J
stop
145
pBG396
(rsT4.12)
S
V
J
stop
166
P215-7
(rsT4.111)
S
V
J
stop
111
P211-11
P214-10
(rsT4.113)
S
V
J
stop
113

FIG. 7B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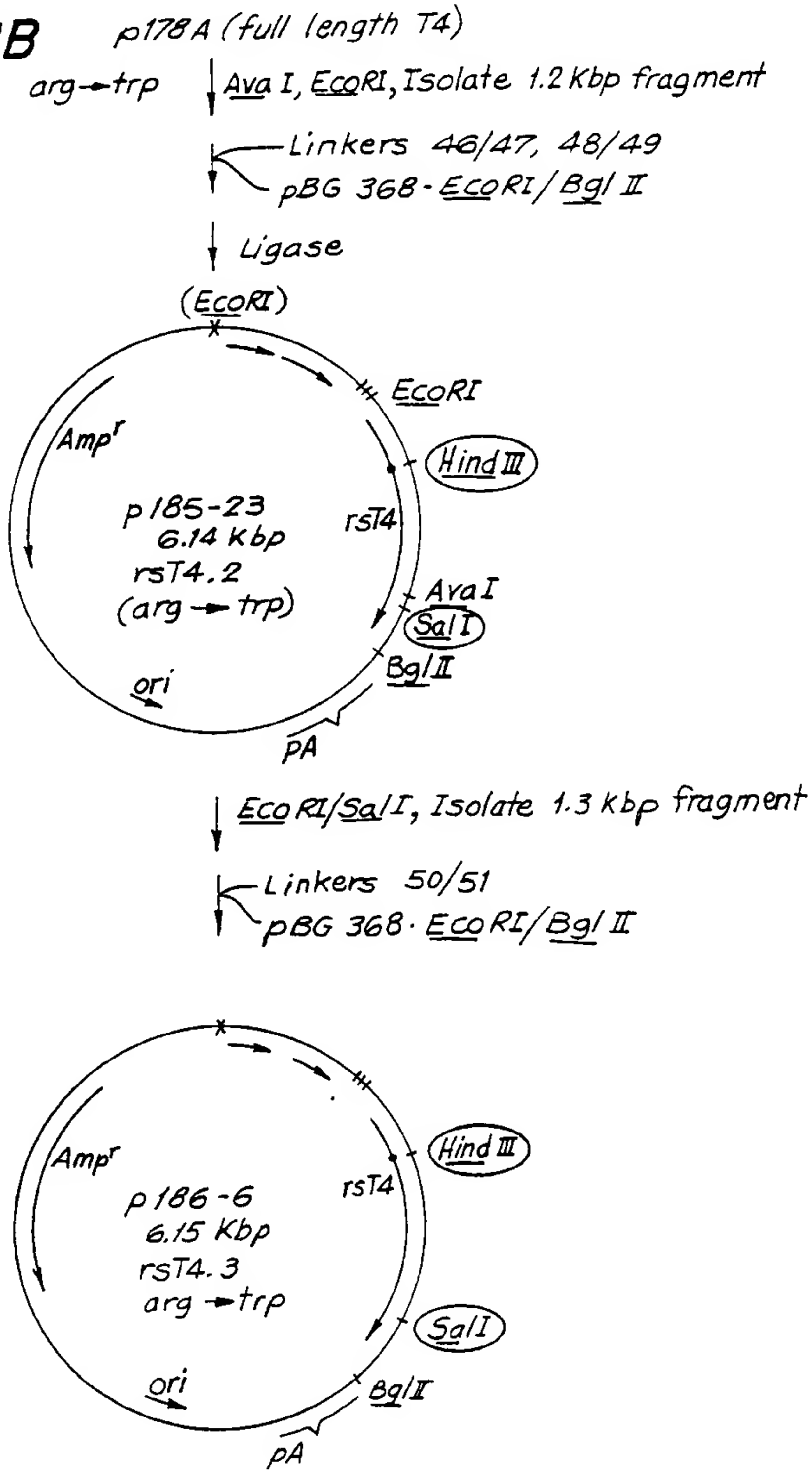
p170-2 (full length T4)
site-directed Mutagenesis
add linker 41
EcoRI, AvaI, isolate 1250 kbp fragment
linkers 34/35
pBG 368 EcoRI/BglII
ligase
EcoRI
XhaI
HindIII
XhoI
EcoRI
HindIII
Amp^r
p178A
7.38 kbp
full length T4
T4
arg → trp
AvaI
NstII
ori
BglII
pA
XhoI
XhoI
p173-51
6.1 kbp
rsT4.1
NstII
BglII
pA
XhoI(partial)/NstII
isolate 1.2 kbp fragment
XhoI/NstII, isolate
4.5 kbp fragment
ligase
EcoRI
HindIII
NheI/NstII
ligase
isolate 6.0kbp
NheI/NstII
isolate 0.675 Kbp
HindIII
pEc100
Amp^r
p181-5
6.1 kbp
rsT4.1
arg → trp
rsT4
NheI
NstII
BglII
ori
pA
Amp^r
pBG 376
6.7 kbp
rsT4.1
arg → trp
ser → phe
rsT4
HindIII
SalI
ori
pA
site-directed Mutagenesis w/linker 55
Amp^r
pBG 377
6.7 kbp
rsT4.1
arg → trp
ser → phe
lys → asn
HindIII
SalI
ori
pA

FIG.8A

FIG. 8B


p178A (full length T4)
arg→trp
Ava I, EcoRI, Isolate 1.2 Kbp fragment
Linkers 46/47, 48/49
pBG 368 · EcoRI/Bgl II
Ligase
(EcoRI)
EcoRI
Ampr
Hind III
p185-23
6.14 kbp
rsT4.2
(arg→trp)
rsT4
Ava I
Sal I
Bgl II
ori
pA
EcoRI/Sal I, Isolate 1.3 kbp fragment
Linkers 50/51
pBG 368 · EcoRI/Bgl II
Ampr
Hind III
p186-6
6.15 Kbp
rsT4.3
arg→trp
rsT4
Sal I
ori
Bgl II
pA

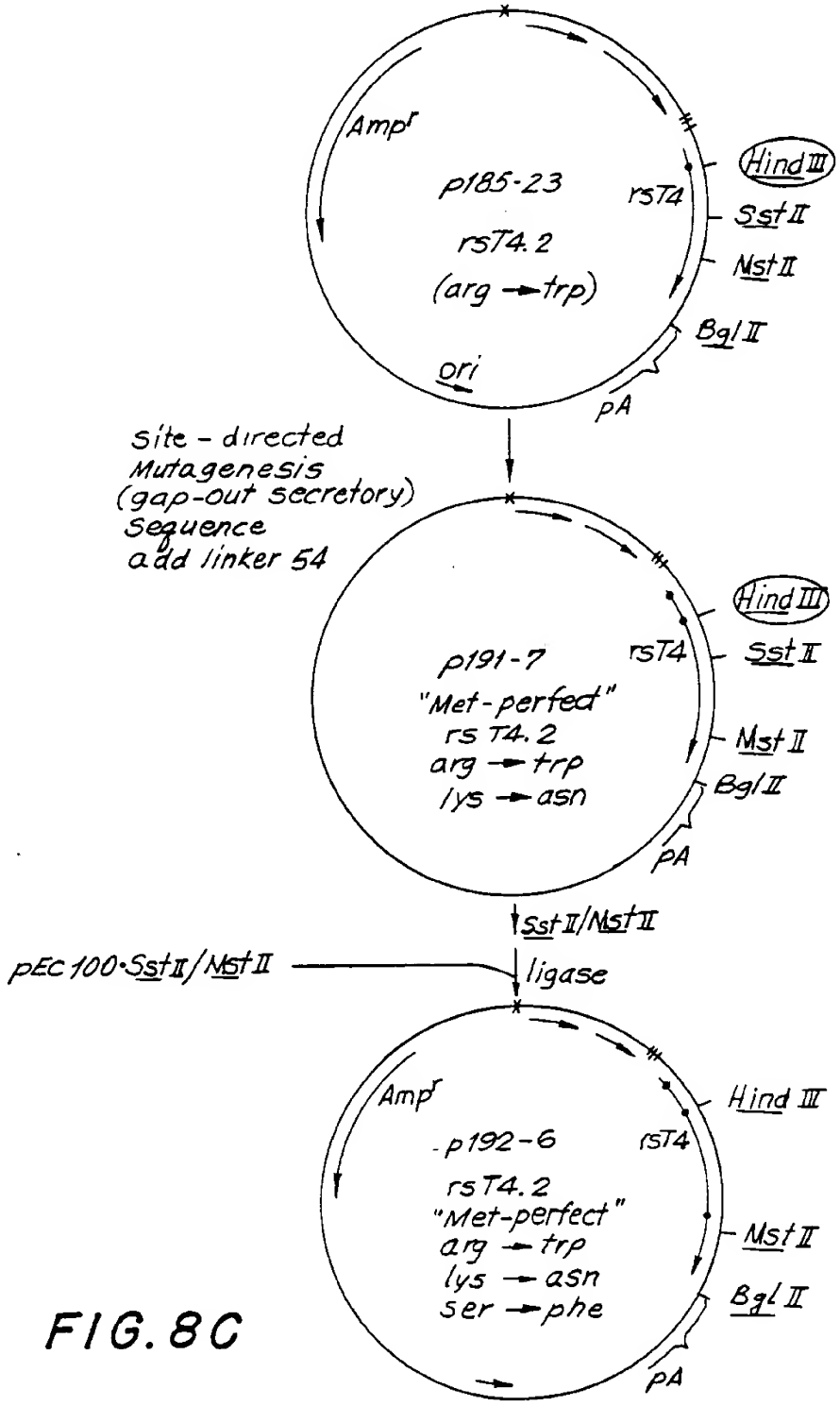

"Met-perfect" cassette for E.coli expression of rsT4
Amp^r
p185-23
rsT4.2
(arg → trp)
rsT4
ori
pA
Hind III
Sst II
Nst II
Bgl II
Site-directed
Mutagenesis
(gap-out secretory)
Sequence
add linker 54
p191-7
"Met-perfect"
rsT4.2
arg → trp
lys → asn
Sst II/Nst II
pEC100·Sst II/Nst II
ligase
p192-6
rsT4.2
"Met-perfect"
arg → trp
lys → asn
ser → phe

FIG. 8C

FIG. 8D

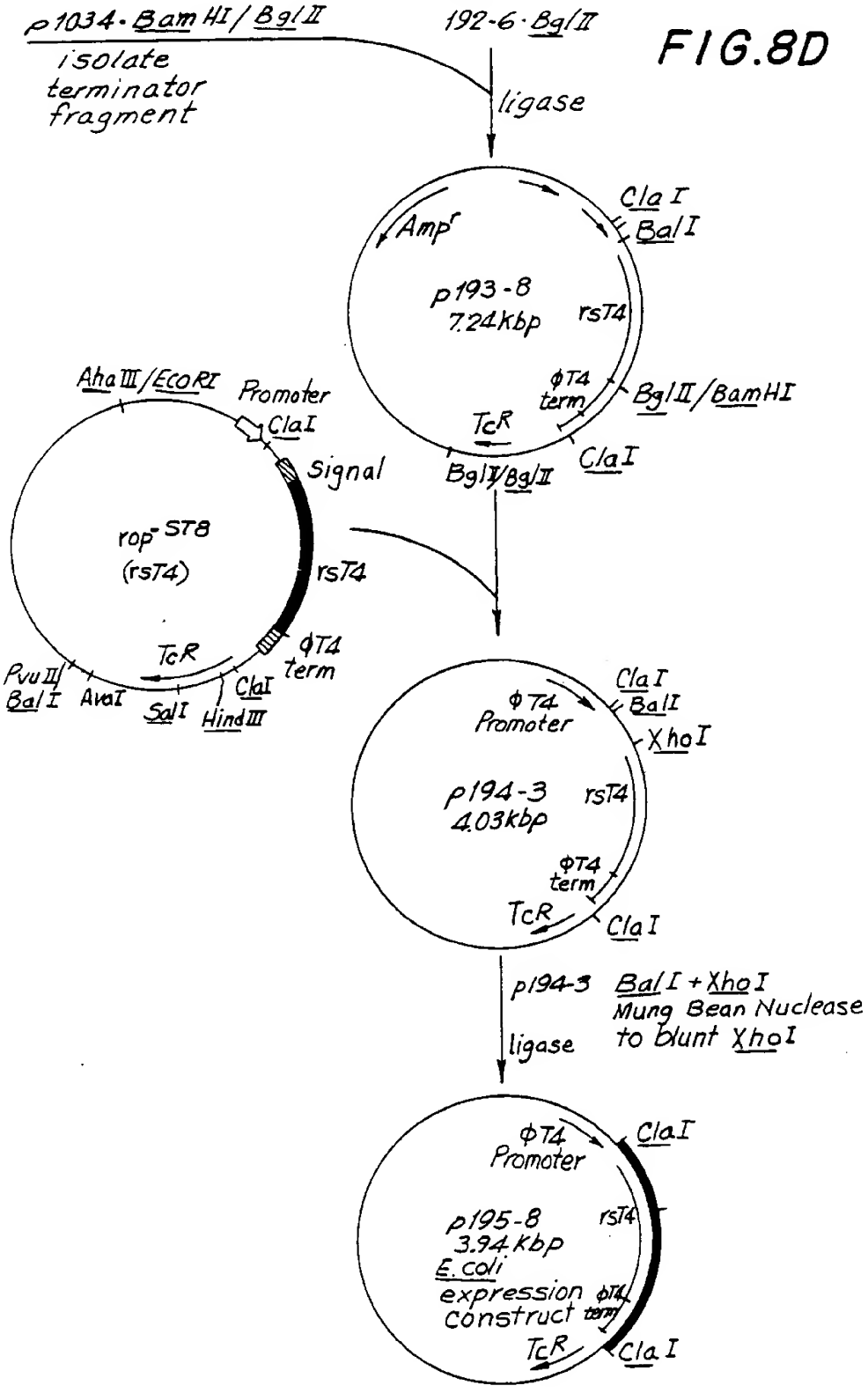
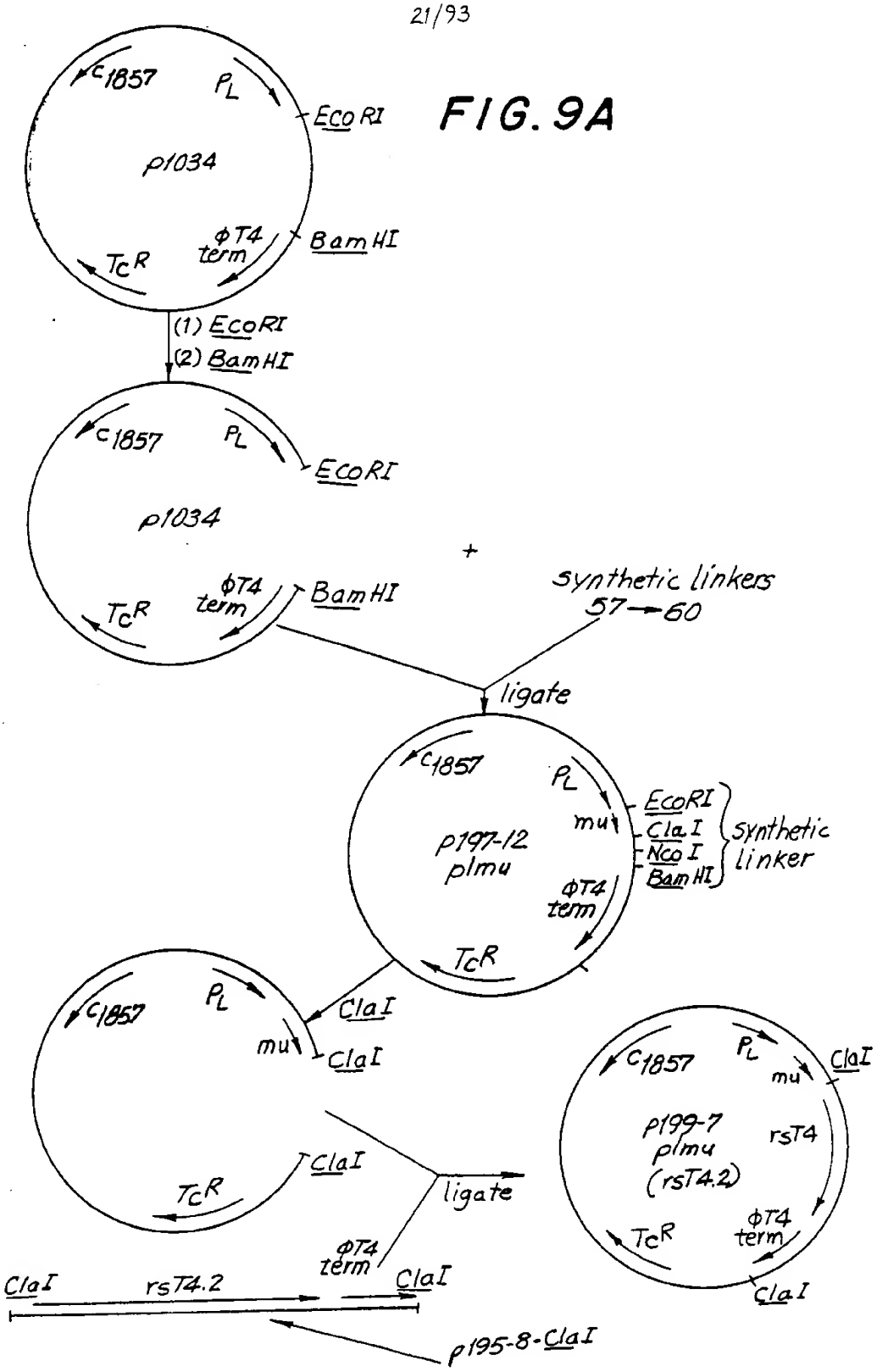

p1034 · BamHI / BglII
isolate terminator fragment
192-6 · BglII
ligase
ClaI
BalI
Ampr
p193-8
7.24kbp
rsT4
φT4 term
BglII/BamHI
TcR
ClaI
BglII/BglII
AhaIII/EcoRI
Promoter
ClaI
Signal
rop- ST8
(rsT4)
rsT4
TcR
φT4 term
PvuII
BalI
AvaI
SalI
ClaI
HindIII
φT4 Promoter
ClaI
BalI
XhoI
p194-3
4.03kbp
rsT4
φT4 term
TcR
ClaI
p194-3
BalI + XhoI
Mung Bean Nuclease
to blunt XhoI
ligase
φT4 Promoter
ClaI
p195-8
3.94 Kbp
E. coli
expression
construct
rsT4
φT4 term
TcR
ClaI

FIG. 9A


c1857
PL
EcoRI
p1034
ϕT4 term
BamHI
TcR
(1) EcoRI
(2) BamHI
synthetic linkers 57→60
ligate
mu
p197-12 plmu
EcoRI
ClaI
NcoI
BamHI
synthetic linker
ClaI
p199-7 plmu (rsT4.2)
rsT4
p195-8-ClaI

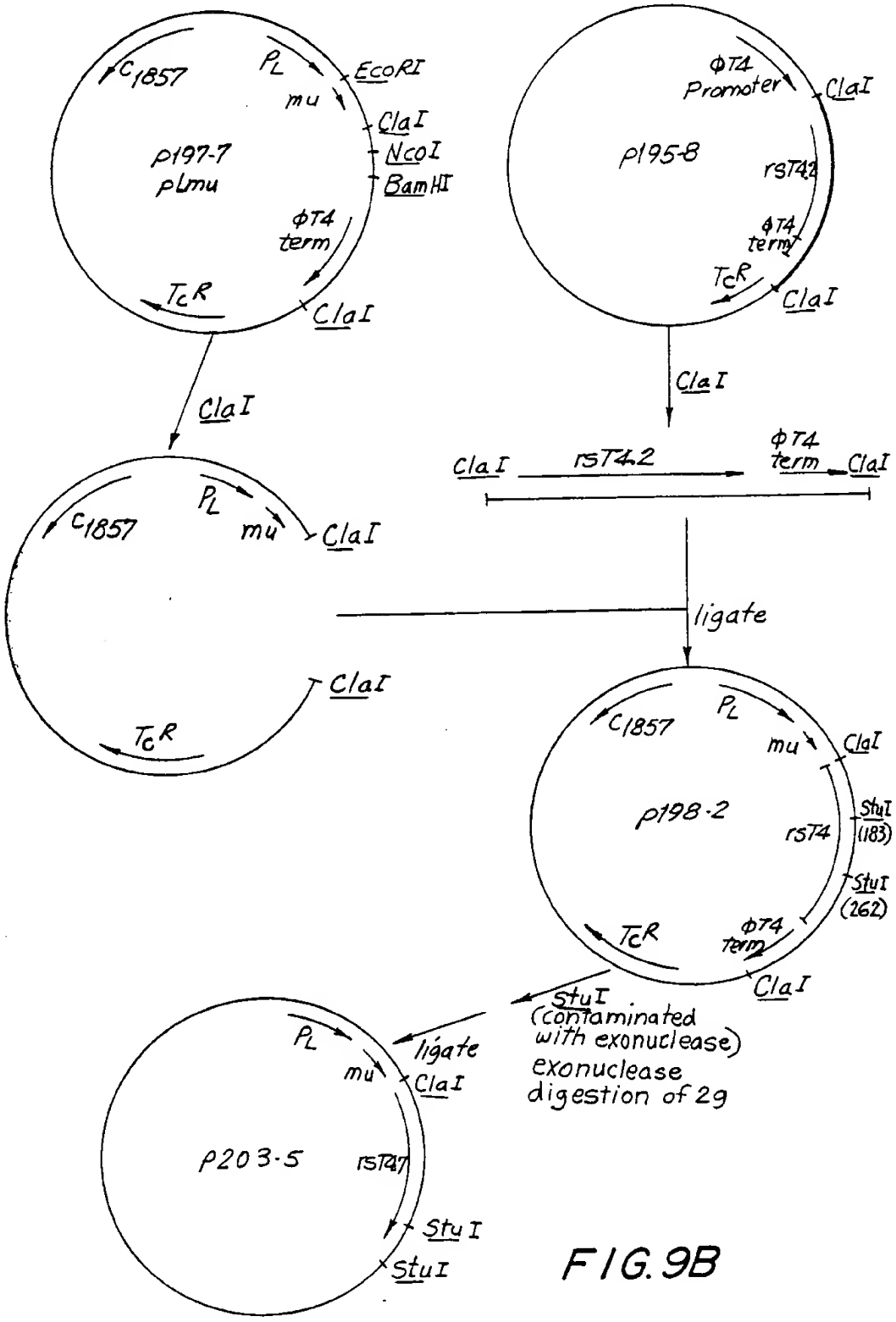

C1857
PL
EcoRI
mu
ClaI
NcoI
BamHI
p197-7
pLmu
φT4
term
TcR
ClaI
ClaI
φT4
Promoter
ClaI
p195-8
rST42
φT4
term
TcR
ClaI
ClaI
ClaI
rST42
φT4
term
ClaI
C1857
PL
mu
ClaI
ClaI
TcR
ligate
C1857
PL
mu
ClaI
p198-2
rST4
StuI
(183)
StuI
(262)
TcR
φT4
term
ClaI
StuI
(contaminated
with exonuclease)
exonuclease
digestion of 2g
PL
mu
ligate
ClaI
p203-5
rST47
StuI
StuI

FIG. 9B

FIG. 9C

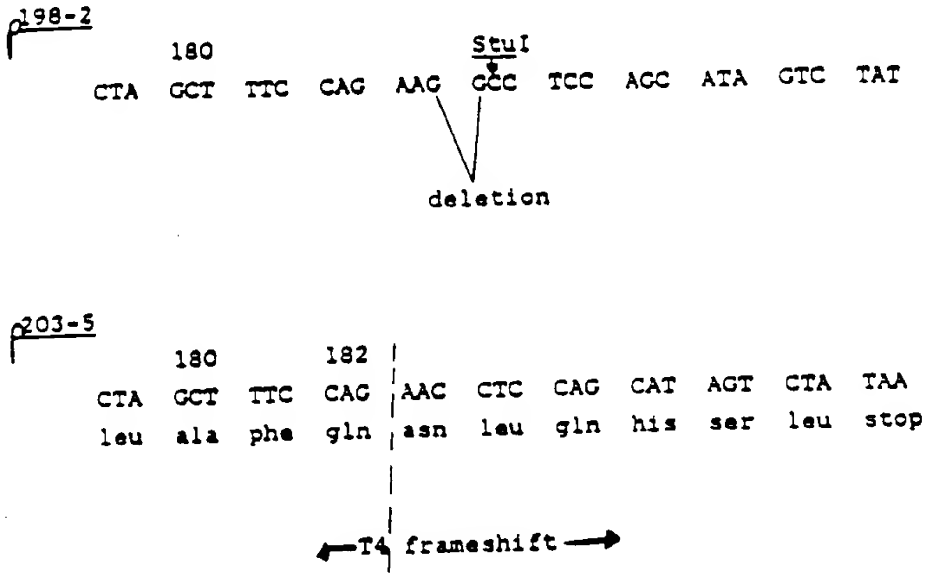

p198-2
180
StuI
CTA GCT TTC CAG AAG GCC TCC AGC ATA GTC TAT
deletion
p203-5
180
182
CTA GCT TTC CAG AAC CTC CAG CAT AGT CTA TAA
leu ala phe gln asn leu gln his ser leu stop
T4 frameshift

FIG. 10

48

5' CTG CCC ACA TGG TCG ACC CCG GTG CAG CCA ATG TGA 3'

49

5' GAT CTC ACA TTG GCT GCA CCG GGG TCG ACC ATG T 3'

50

5' TCG ACC CCG GTG CAG CCA ATG GCC CTG ATT TGA 3'

3' GG GGC CAC GTC GGT TAC CGG GAC TAA ACT CTA G 5'

51

41

5' GAA GAA GCT TGT GGG ACC AAG 3'

34

5' TCG GGA CAG GTC CTG CTG GAA TCC AAC ATC AAG TGA A 3'

3' CTG TCC AGG ACG ACC TTA GGT TGT AGT TCA CTT CTA G 5'

35

55

5' CAG CCA CCC AAG GAA ACA AAG TGG 3'

46

5' TCG GGA CAG GTC CTG CTG GAA TCC AAC ATC AAG GTT 3'

47

5' GGG CAG AAC CTT GAT GTT GGA TTC CAG CAG GAC CTG TC 3'

FIG. 10(cont'd)

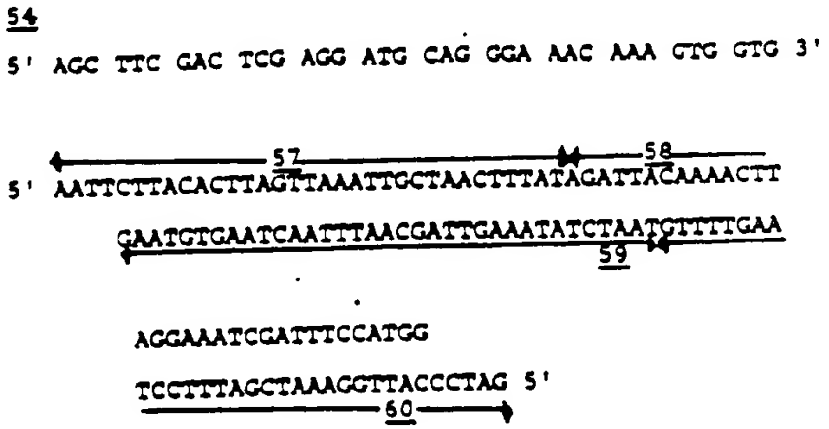

54
5' AGC TTC GAC TCG AGG ATG CAG GGA AAC AAA GTG GTG 3'
57
58
5' AATTCTTACACTTAGTTAAATTGCTAACTTTATAGATTACAAAACTT
GAATGTGAATCAATTTAACGATTGAAATATCTAATGTTTTGAA
59
AGGAAATCGATTTCCATGG
TCCTTTAGCTAAAGGTTACCCTAG 5'
60

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FIG.11

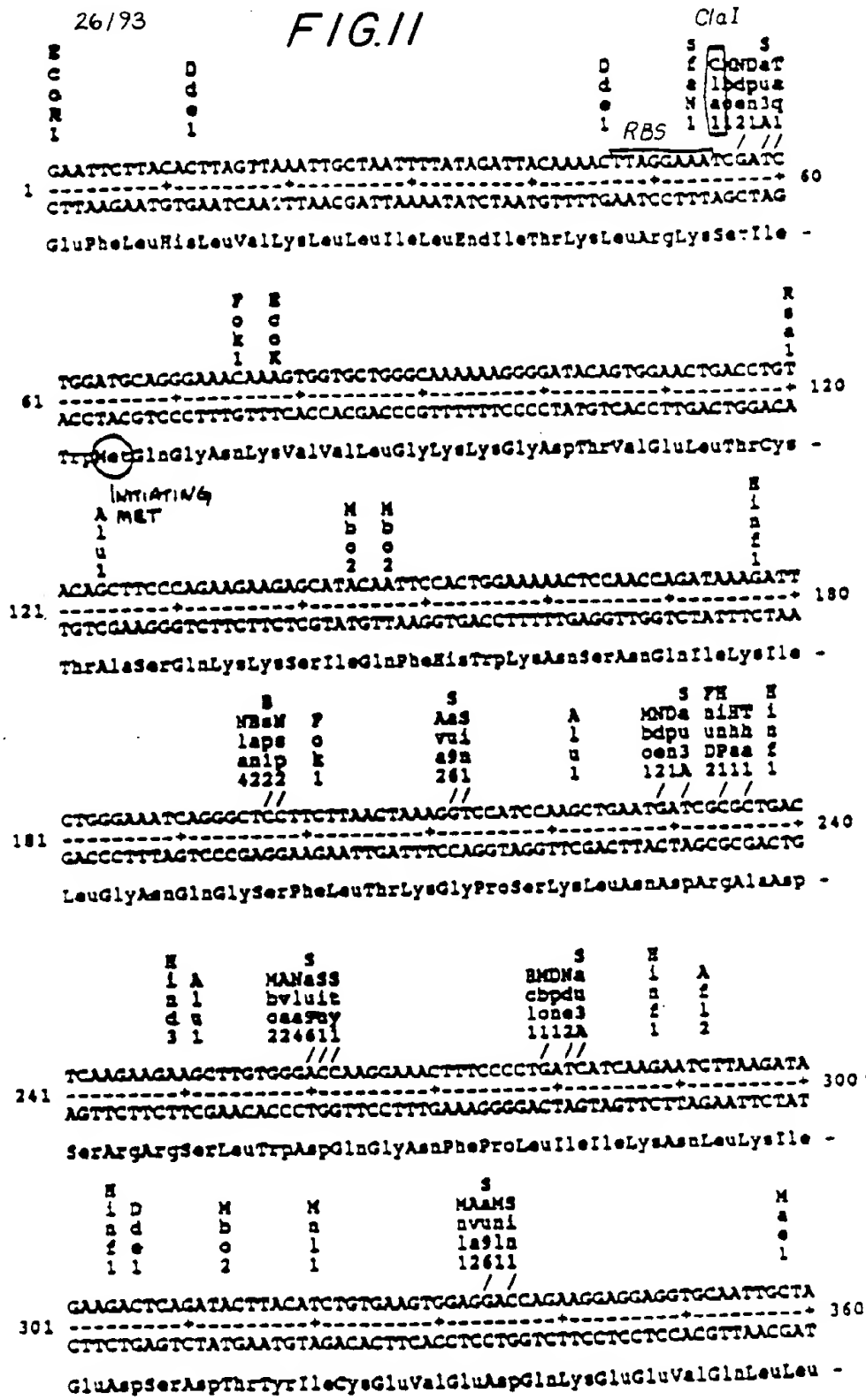

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FIG. 11(cont'd)

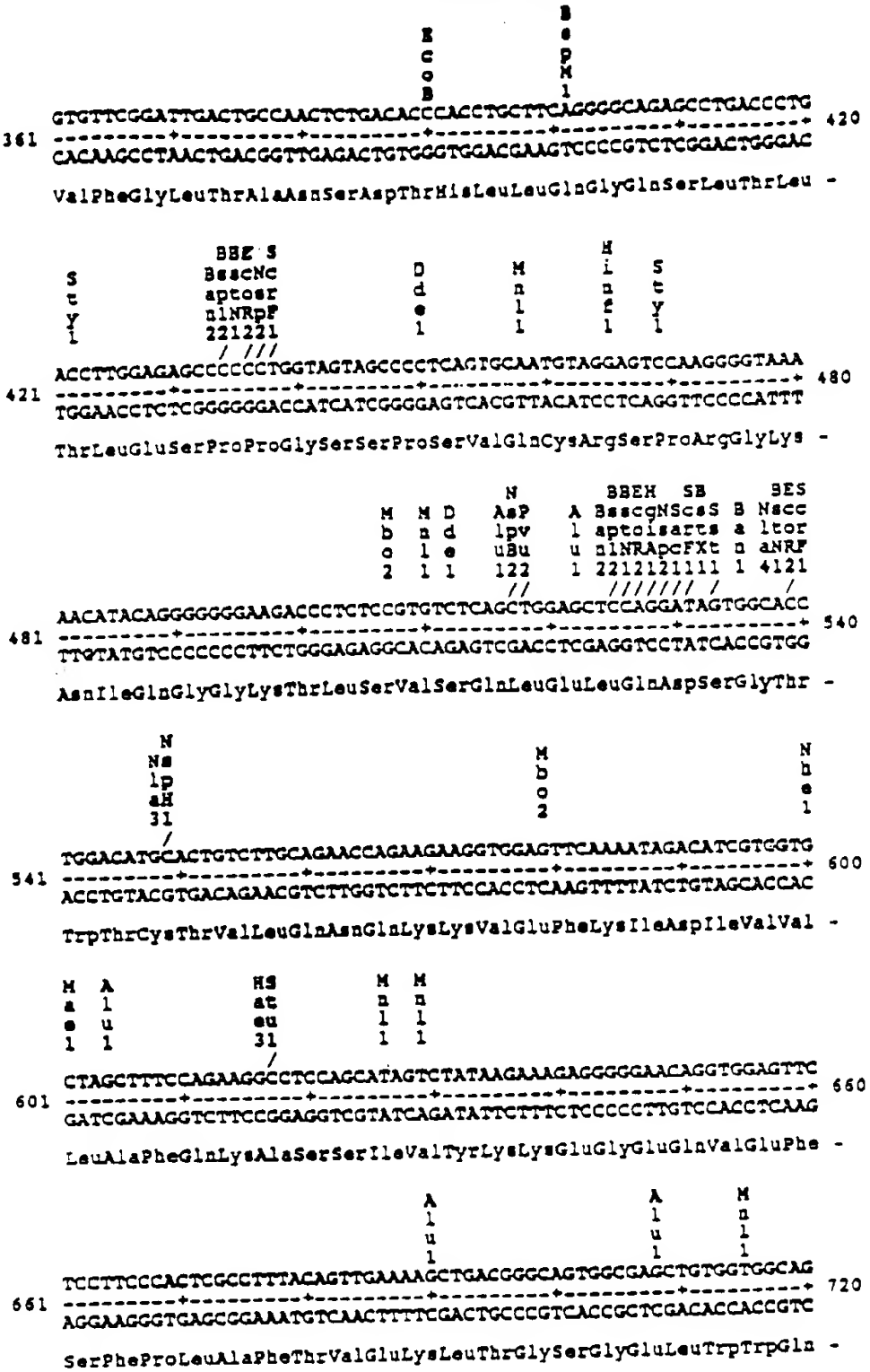

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FIG.11(cont'd)

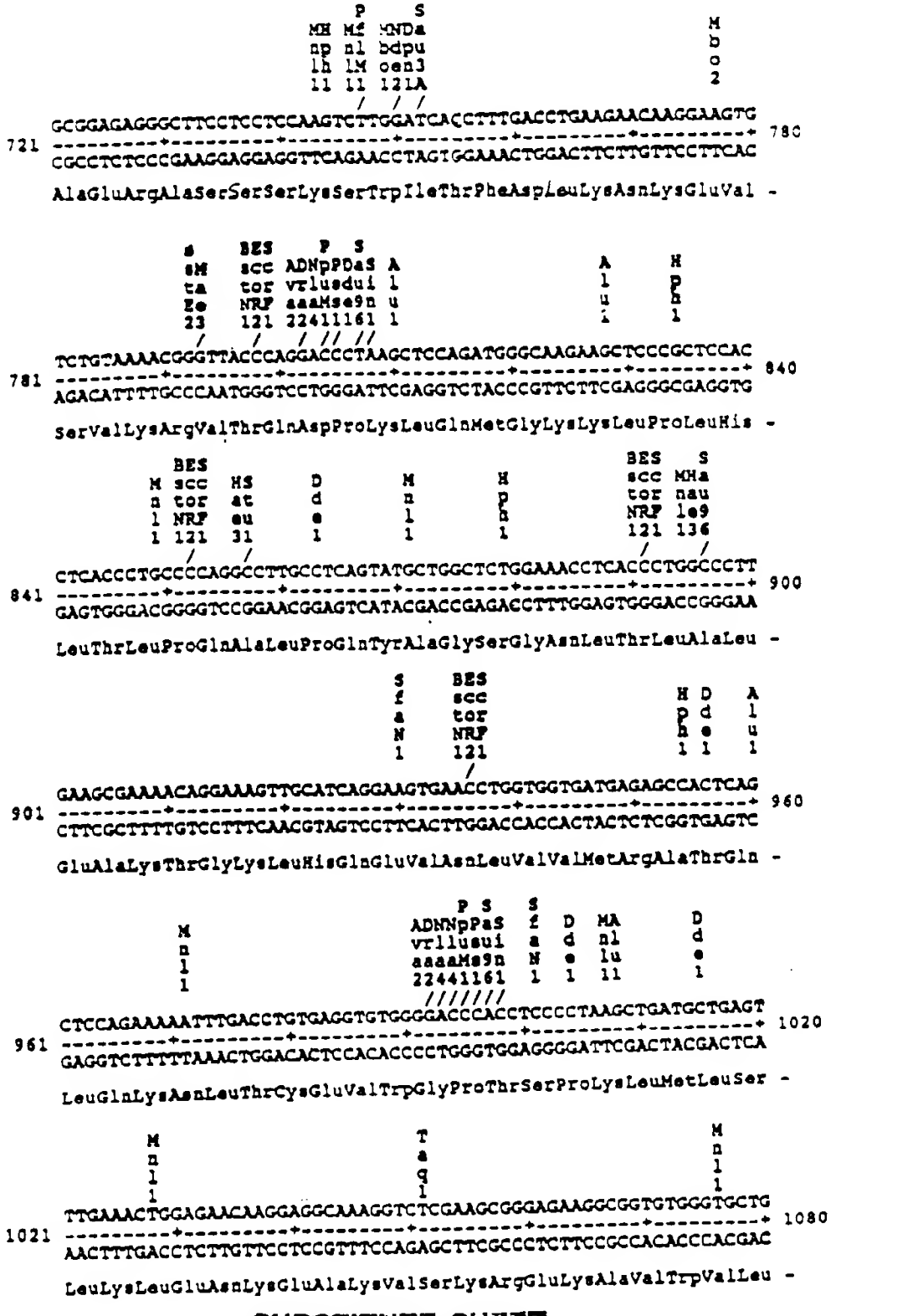

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FIG. 11(cont'd)

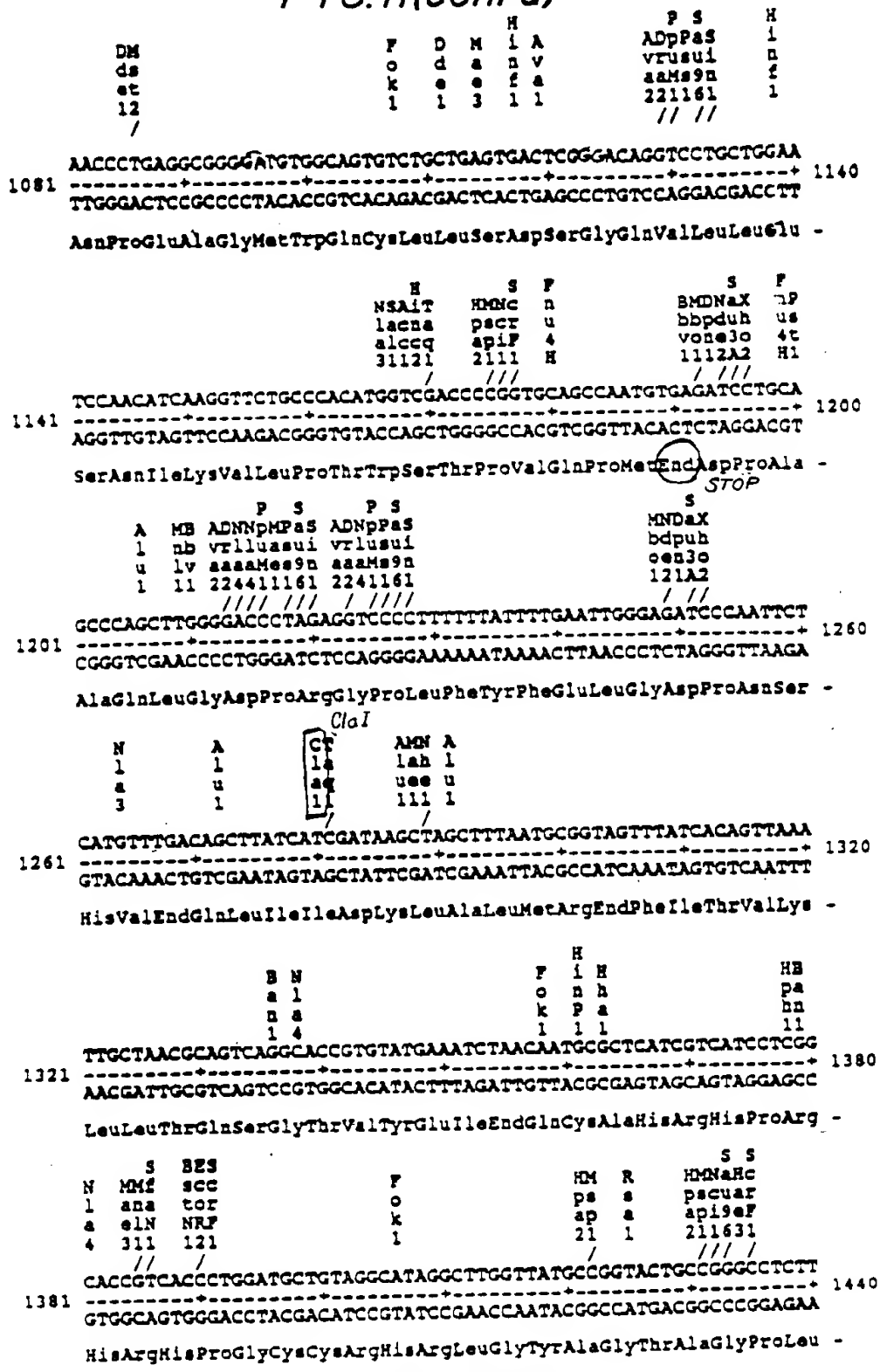

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FIG. 11(cont'd)

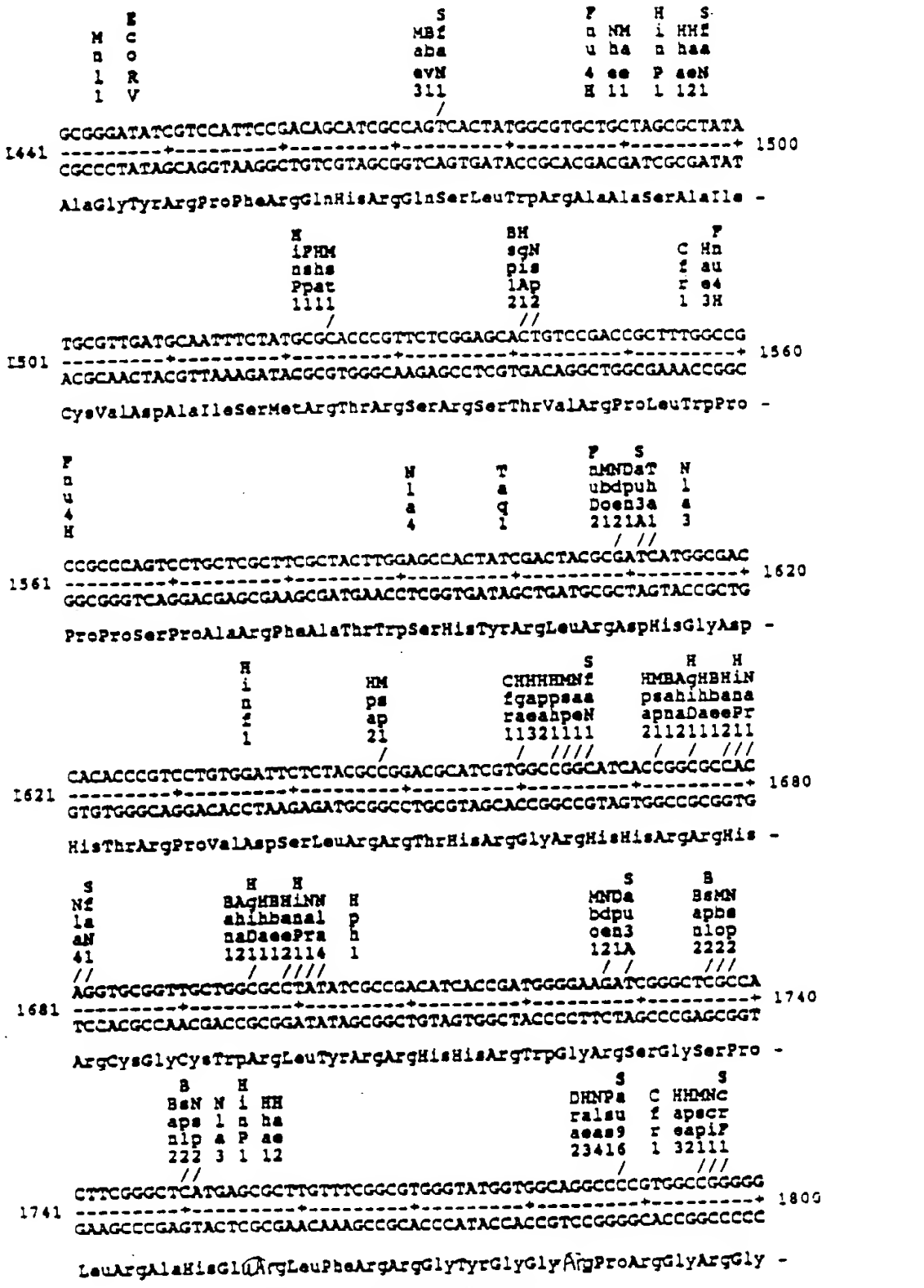

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FIG. 11(cont'd)

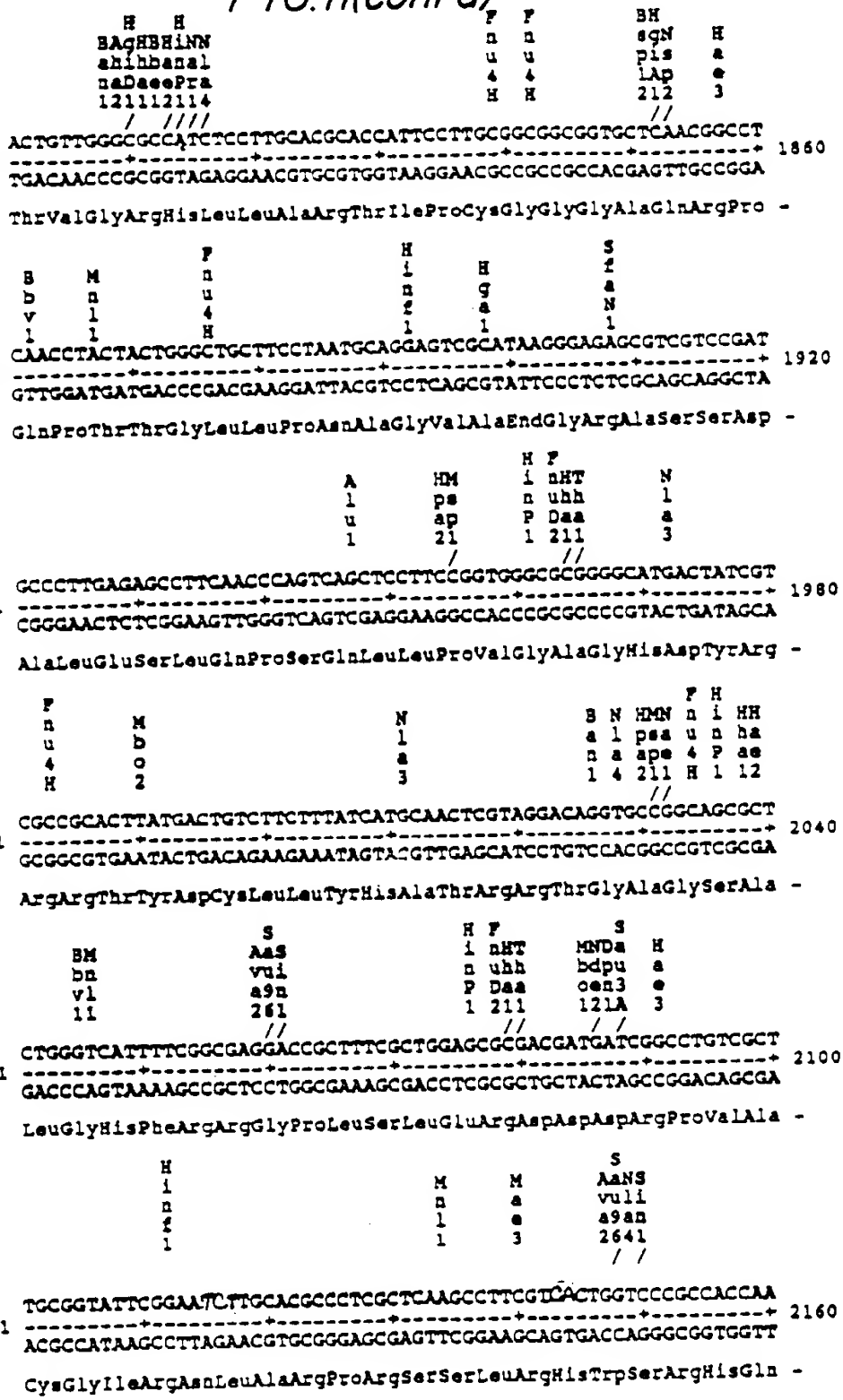

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FIG. 11(cont'd)

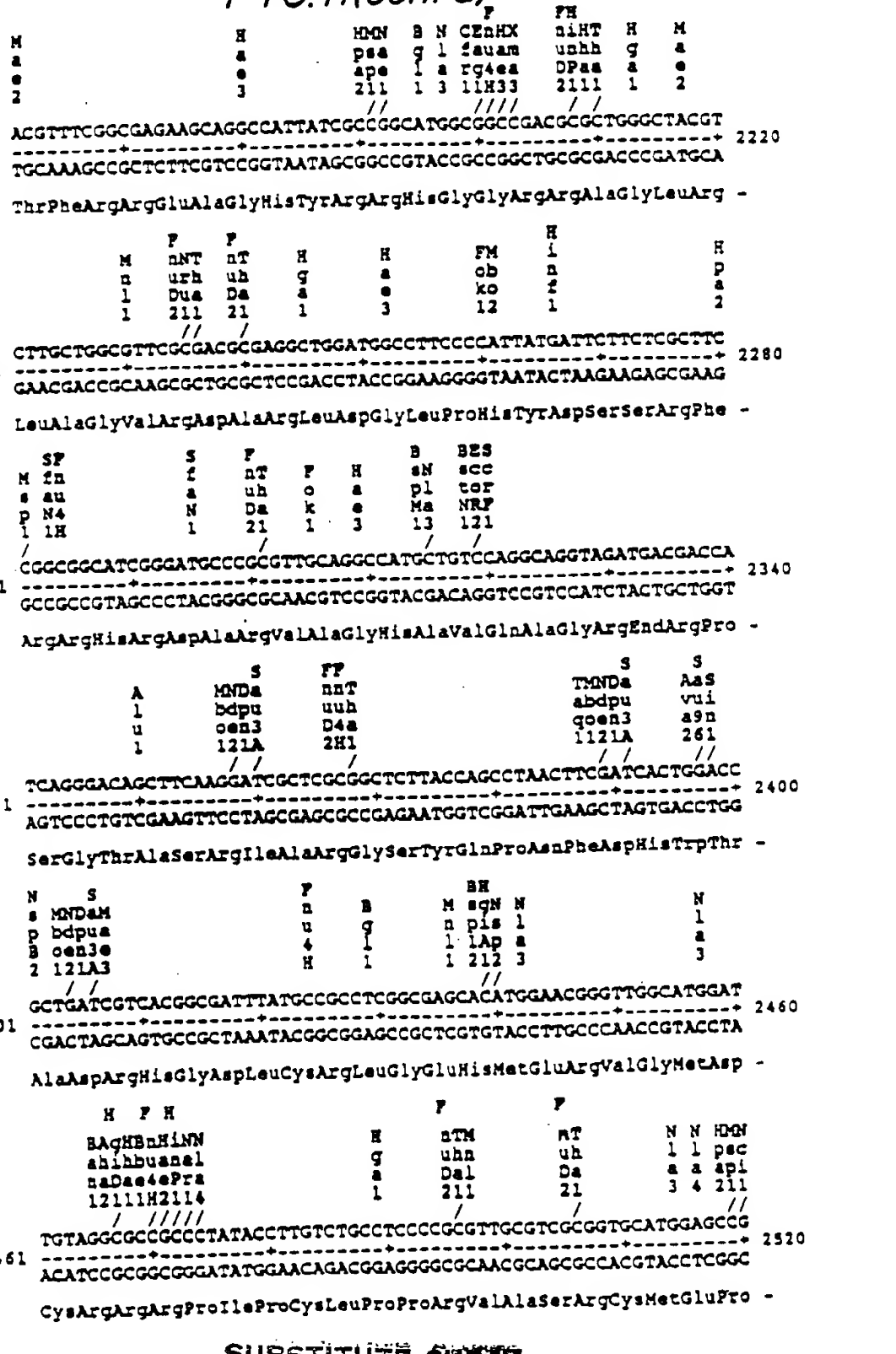

FIG. 11(cont'd)

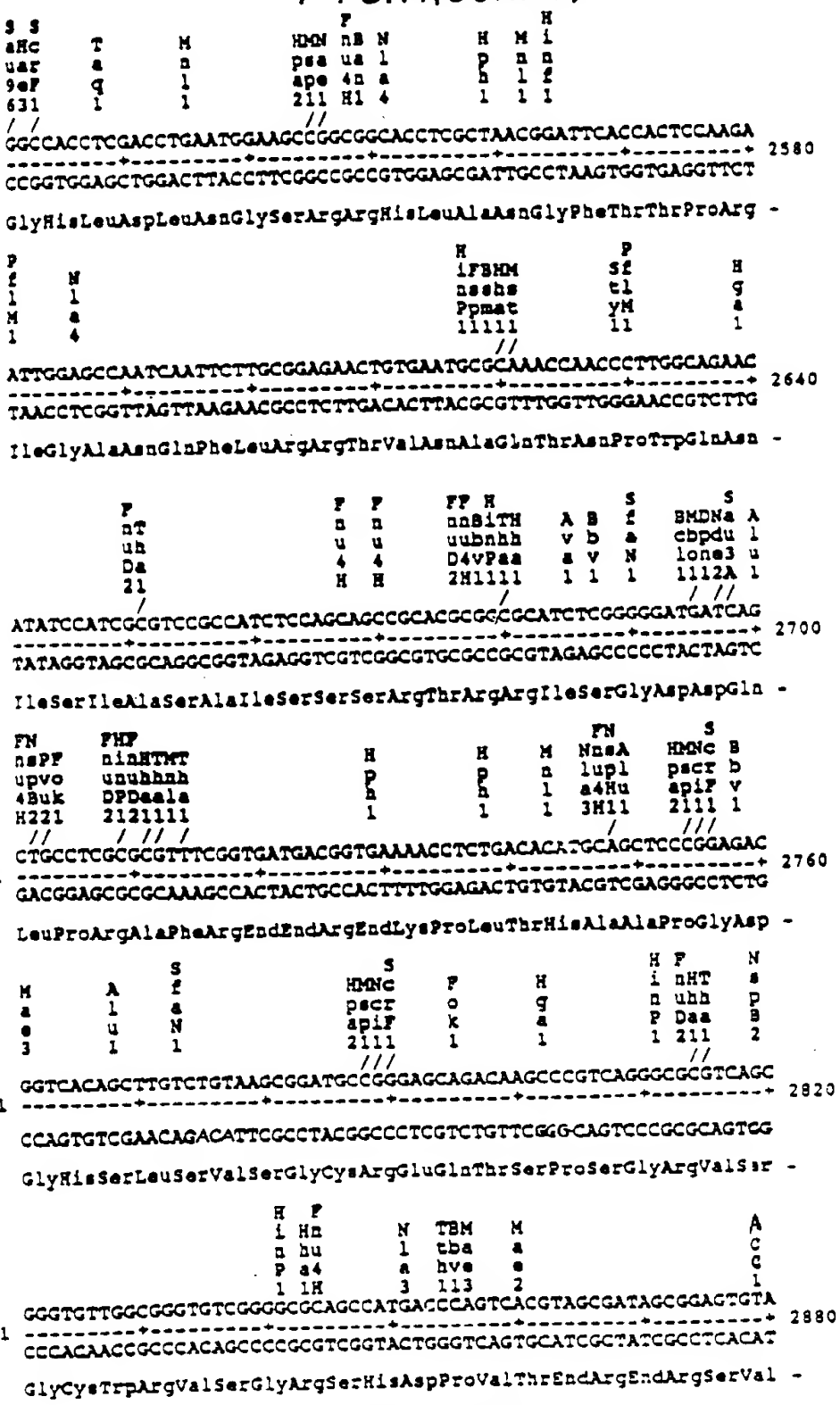
GGCCACCTCGACCTGAATGGAAGCCGGCGGCACCTCGCTAACGGATTCACCACTCCAAGA
2521 2580
CCGGTGGAGCTGGACTTACCTTCGGCCGCCGTGGAGCGATTGCCTAAGTGGTGAGGTTCT
GlyHisLeuAspLeuAsnGlySerArgArgHisLeuAlaAsnGlyPheThrThrProArg -
ATTGGAGCCAATCAATTCTTGCGGAGAACTGTGAATGCGCAAACCAACCCTTGGCAGAAC
2581 2640
TAACCTCGGTTAGTTAAGAACGCCTCTTGACACTTACGCGTTTGGTTGGGAACCGTCTTG
IleGlyAlaAsnGlnPheLeuArgArgThrValAsnAlaGlnThrAsnProTrpGlnAsn -
ATATCCATCGCGTCCGCCATCTCCAGCAGCCGCACGCGCGCGCATCTCGGGGGATGATCAG
2641 2700
TATAGGTAGCGCAGGCGGTAGAGGTCGTCGGCGTGCGCCGCGTAGAGCCCCCTACTAGTC
IleSerIleAlaSerAlaIleSerSerSerArgThrArgArgIleSerGlyAspAspGln -
CTGCCTCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGAC
2701 2760
GACGGAGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTG
LeuProArgAlaPheArgEndEndArgEndLysProLeuThrHisAlaAlaProGlyAsp -
GGTCACAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGC
2761 2820
CCAGTGTCGAACAGACATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCG
GlyHisSerLeuSerValSerGlyCysArgGluGlnThrSerProSerGlyArgValSer -
GGGTGTTGGCGGGTGTCGGGGCGCAGCCATGACCCAGTCACGTAGCGATAGCGGAGTGTA
2821 2880
CCCACAACCGCCCACAGCCCCGCGTCGGTACTGGGTCAGTGCATCGCTATCGCCTCACAT
GlyCysTrpArgValSerGlyArgSerHisAspProValThrEndArgEndArgSerVal -

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FIG. 11(cont'd)

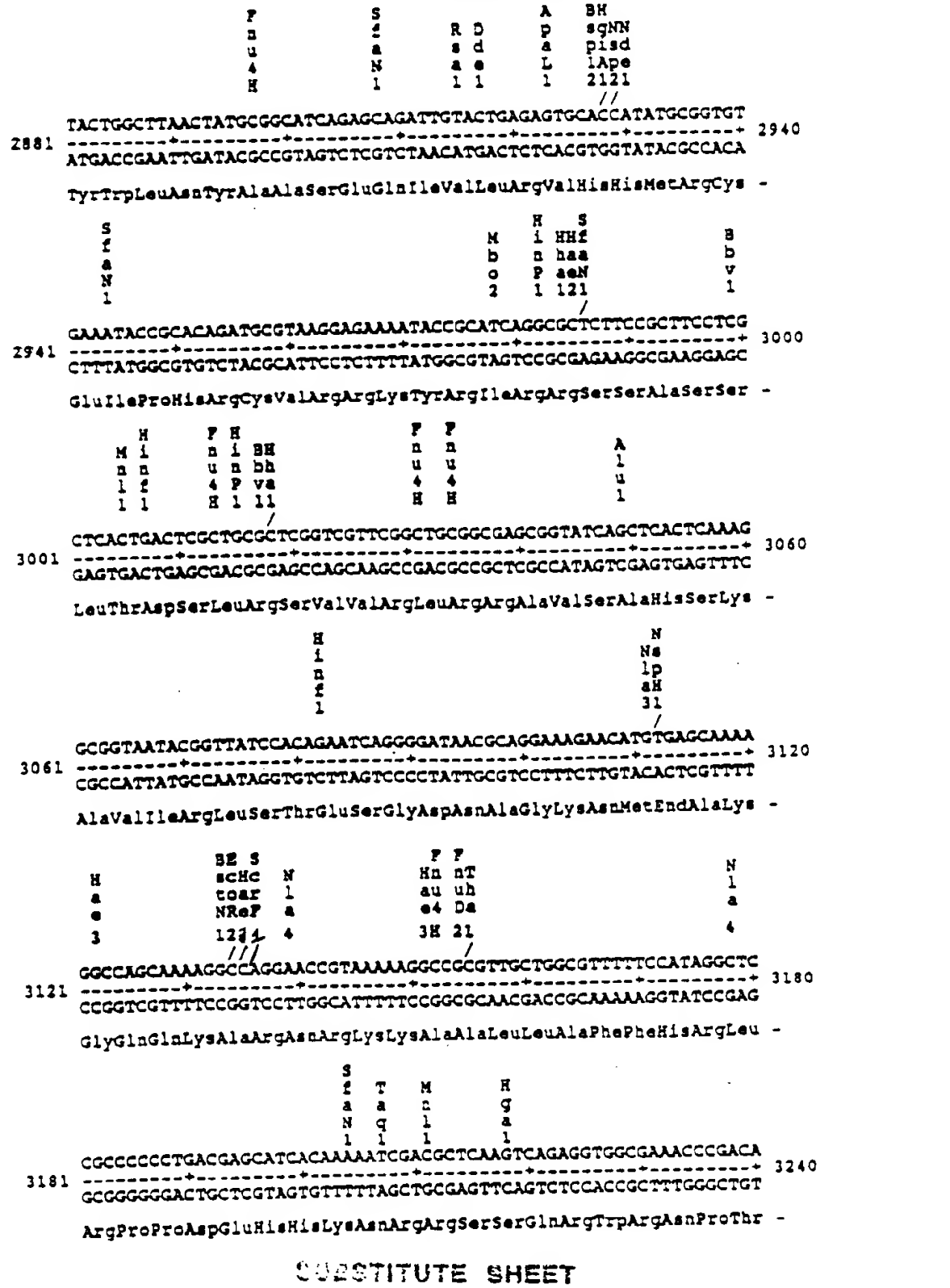

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FIG. 11(cont'd)

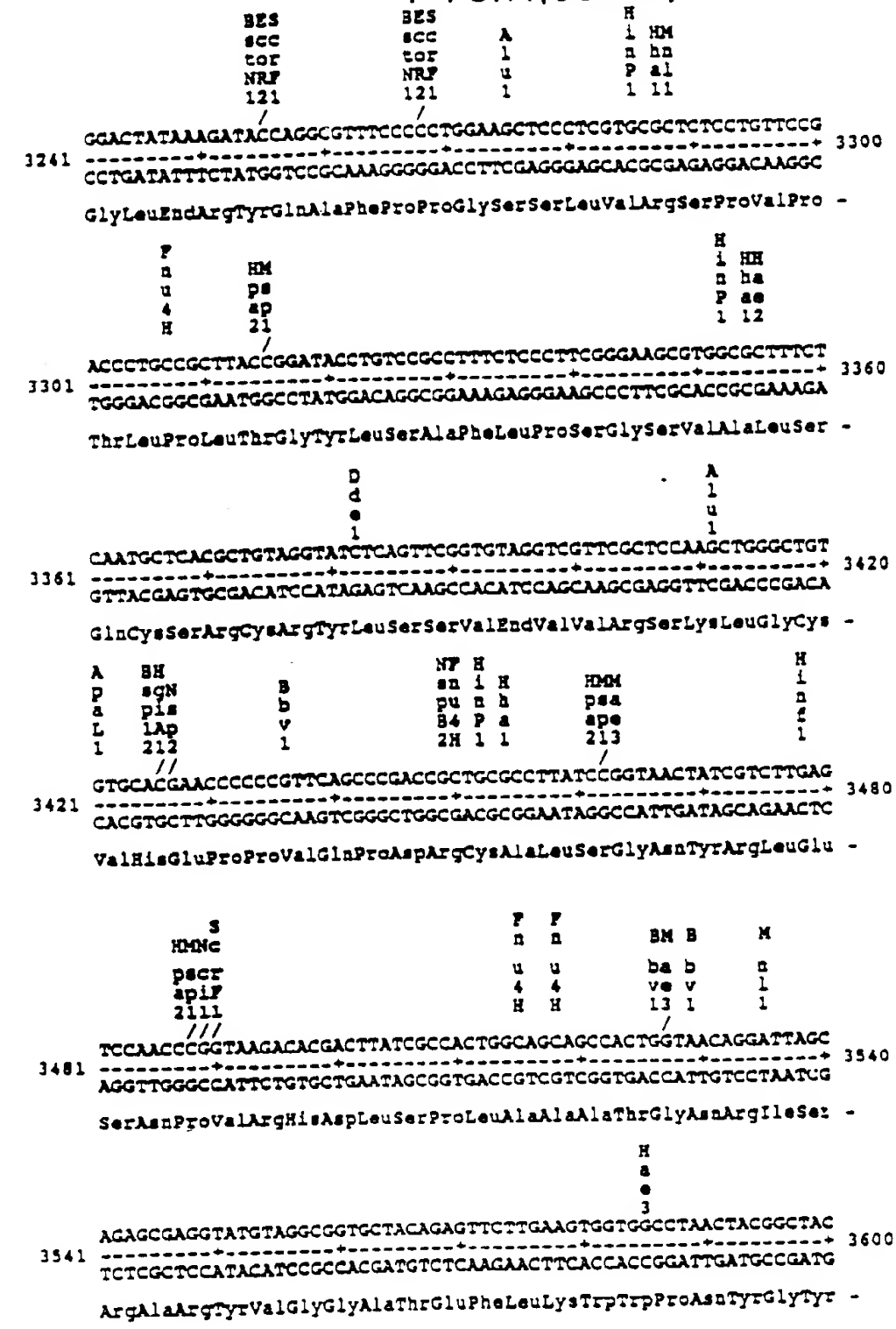

FIG. 11(cont'd)

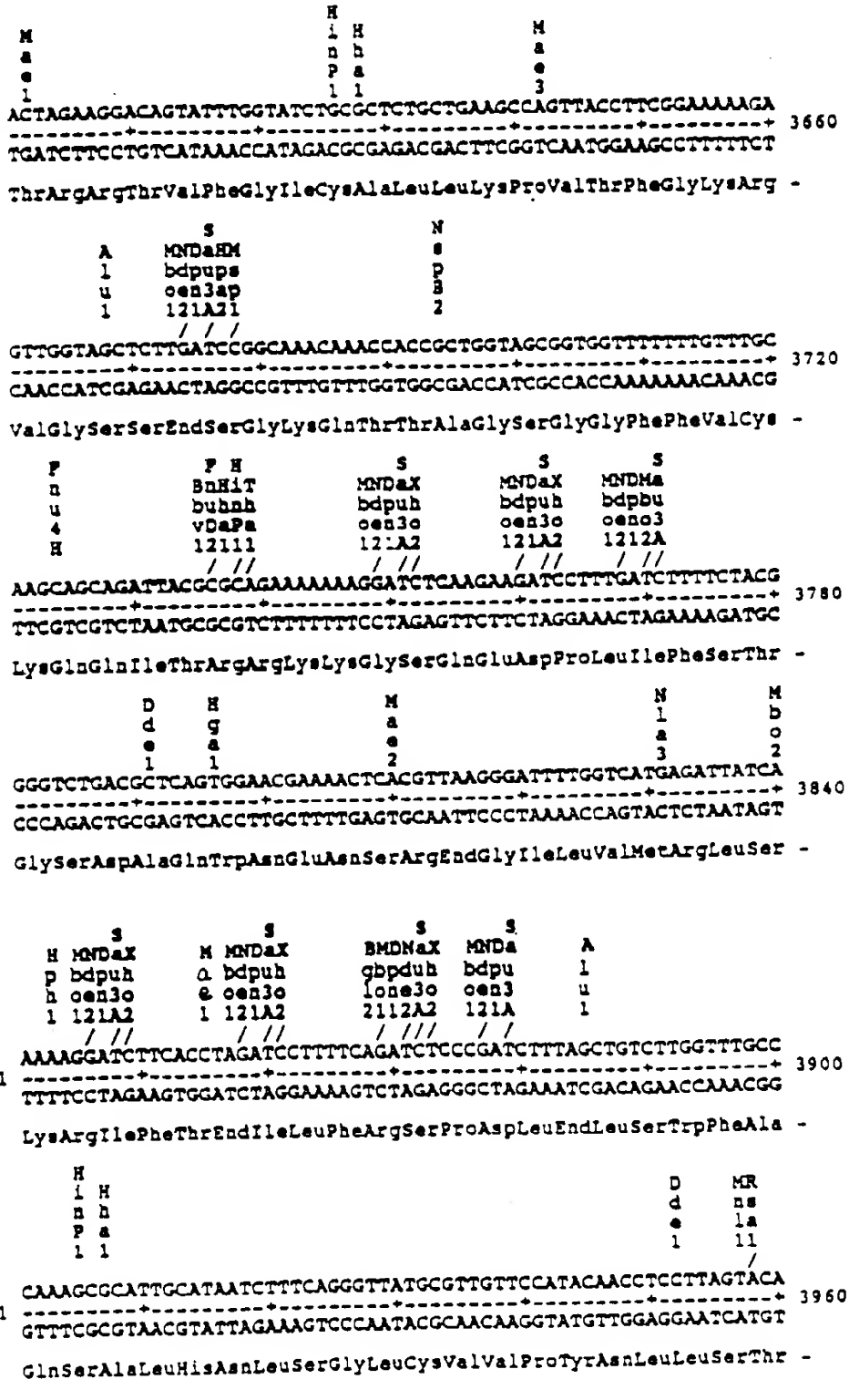
ACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGA
1601 3660
TGATCTTCCTGTCATAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTCT
ThrArgArgThrValPheGlyIleCysAlaLeuLeuLysProValThrPheGlyLysArg -
GTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGC
1661 3720
CAACCATCGAGAACTAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACG
ValGlySerSerEndSerGlyLysGlnThrThrAlaGlySerGlyGlyPhePheValCys -
AAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACG
3721 3780
TTCGTCGTCTAATGCGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAACTAGAAAAGATGC
LysGlnGlnIleThrArgArgLysLysGlySerGlnGluAspProLeuIlePheSerThr -
GGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCA
3781 3840
CCCAGACTGCGAGTCACCTTGCTTTTGAGTGCAATTCCCTAAAACCAGTACTCTAATAGT
GlySerAspAlaGlnTrpAsnGluAsnSerArgEndGlyIleLeuValMetArgLeuSer -
AAAAGGATCTTCACCTAGATCCTTTTCAGATCTCCCGATCTTTAGCTGTCTTGGTTTGCC
3841 3900
TTTTCCTAGAAGTGGATCTAGGAAAAGTCTAGAGGGCTAGAAATCGACAGAACCAAACGG
LysArgIlePheThrEndIleLeuPheArgSerProAspLeuEndLeuSerTrpPheAla -
CAAAGCGCATTGCATAATCTTTCAGGGTTATGCGTTGTTCCATACAACCTCCTTAGTACA
3901 3960
GTTTCGCGTAACGTATTAGAAAGTCCCAATACGCAACAAGGTATGTTGGAGGAATCATGT
GlnSerAlaLeuHisAsnLeuSerGlyLeuCysValValProTyrAsnLeuLeuSerThr -

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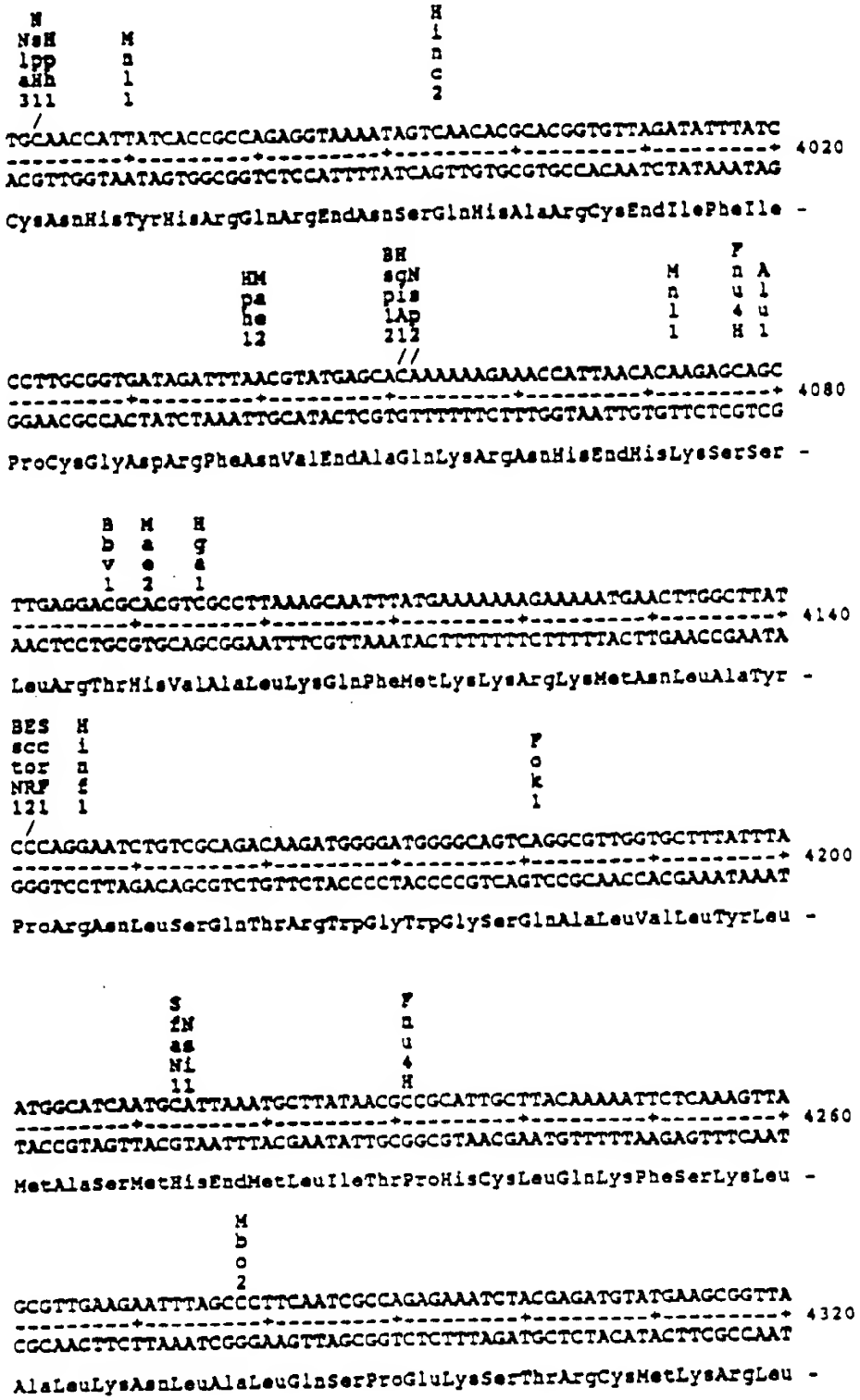
FIG. 11(cont'd)



SUBSTITUTE SHEET

FIG. 11(cont'd)

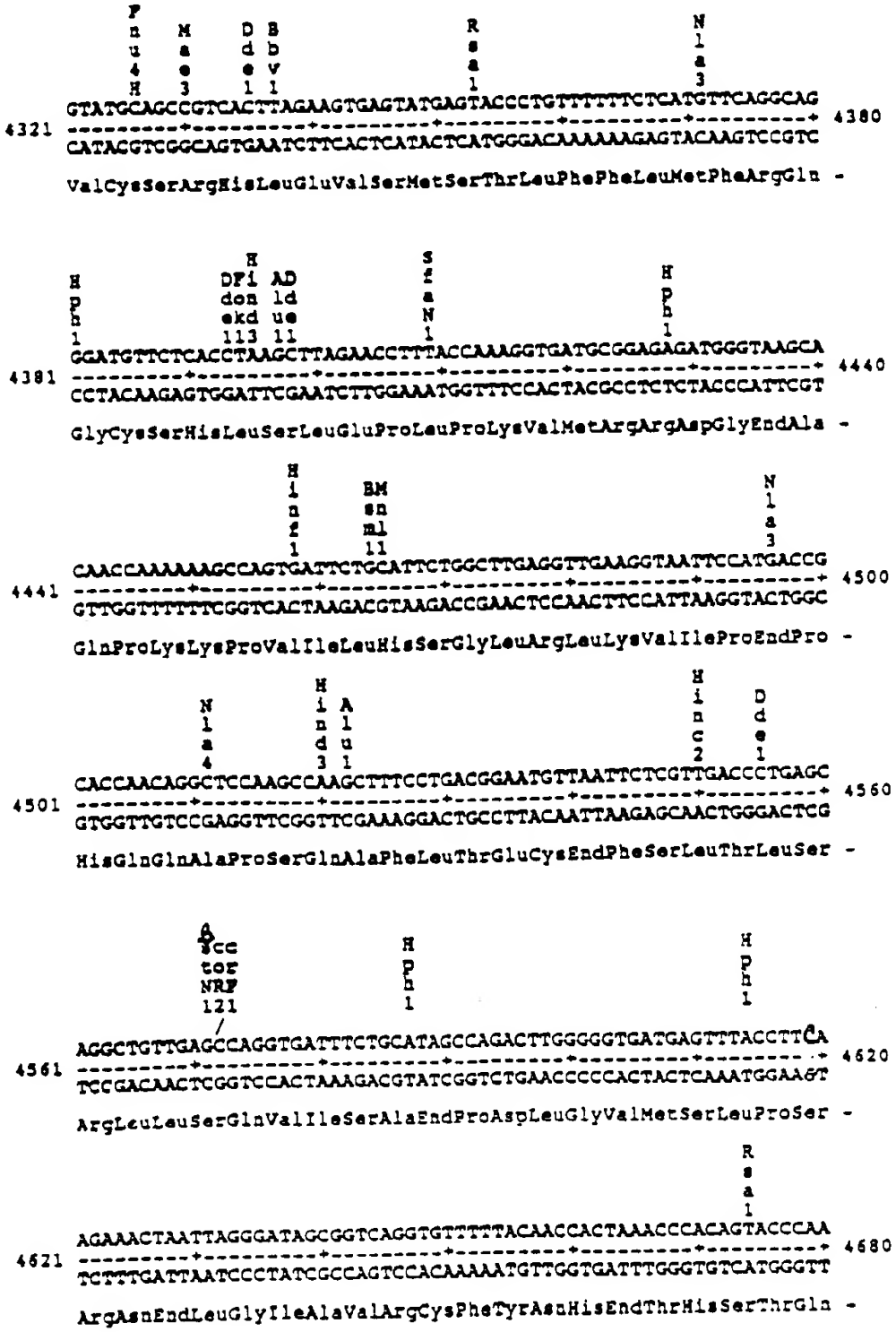

FnuDH 4 3
MaeIII 3
DdeI 1
BbvI 1
RsaI 1
NlaIII 3
GTATGCAGCCGTCACTTAGAAGTGAGTATGAGTACCCTGTTTTTTCTCATGTTCAGGCAG
CATACGTCGGCAGTGAATCTTCACTCATACTCATGGGACAAAAAAGAGTACAAGTCCGTC
4321
4380
ValCysSerArgHisLeuGluValSerMetSerThrLeuPhePheLeuMetPheArgGln -
HphI 1
HinfI 113
DraIII 11
SfaNI 1
HphI 1
GGATGTTCTCACCTAAGCTTAGAACCTTTACCAAAGGTGATGCGGAGAGATGGGTAAGCA
CCTACAAGAGTGGATTCGAATCTTGGAAATGGTTTCCACTACGCCTCTCTACCCATTCGT
4381
4440
GlyCysSerHisLeuSerLeuGluProLeuProLysValMetArgArgAspGlyEndAla -
HinfI 1
BMSmaI 11
NlaIII 3
CAACCAAAAAAGCCAGTGATTCTGCATTCTGGCTTGAGGTTGAAGGTAATTCCATGACCG
GTTGGTTTTTTCGGTCACTAAGACGTAAGACCGAACTCCAACTTCCATTAAGGTACTGGC
4441
4500
GlnProLysLysProValIleLeuHisSerGlyLeuArgLeuLysValIleProEndPro -
NlaIV 4
HindIII 1
AluI 1
HincII 2
DdeI 1
CACCAACAGGCTCCAAGCCAAGCTTTCCTGACGGAATGTTAATTCTCGTTGACCCTGAGC
GTGGTTGTCCGAGGTTCGGTTCGAAAGGACTGCCTTACAATTAAGAGCAACTGGGACTCG
4501
4560
HisGlnGlnAlaProSerGlnAlaPheLeuThrGluCysEndPheSerLeuThrLeuSer -
AvaII NRF 121
HphI 1
HphI 1
AGGCTGTTGAGCCAGGTGATTTCTGCATAGCCAGACTTGGGGGTGATGAGTTTACCTTCA
TCCGACAACTCGGTCCACTAAAGACGTATCGGTCTGAACCCCCACTACTCAAATGGAAGT
4561
4620
ArgLeuLeuSerGlnValIleSerAlaEndProAspLeuGlyValMetSerLeuProSer -
RsaI 1
AGAAACTAATTAGGGATAGCGGTCAGGTGTTTTTACAACCACTAAACCCACAGTACCCAA
TCTTTGATTAATCCCTATCGCCAGTCCACAAAAATGTTGGTGATTTGGGTGTCATGGGTT
4621
4680
ArgAsnEndLeuGlyIleAlaValArgCysPheTyrAsnHisEndThrHisSerThrGln -

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FIG. 11(cont'd)

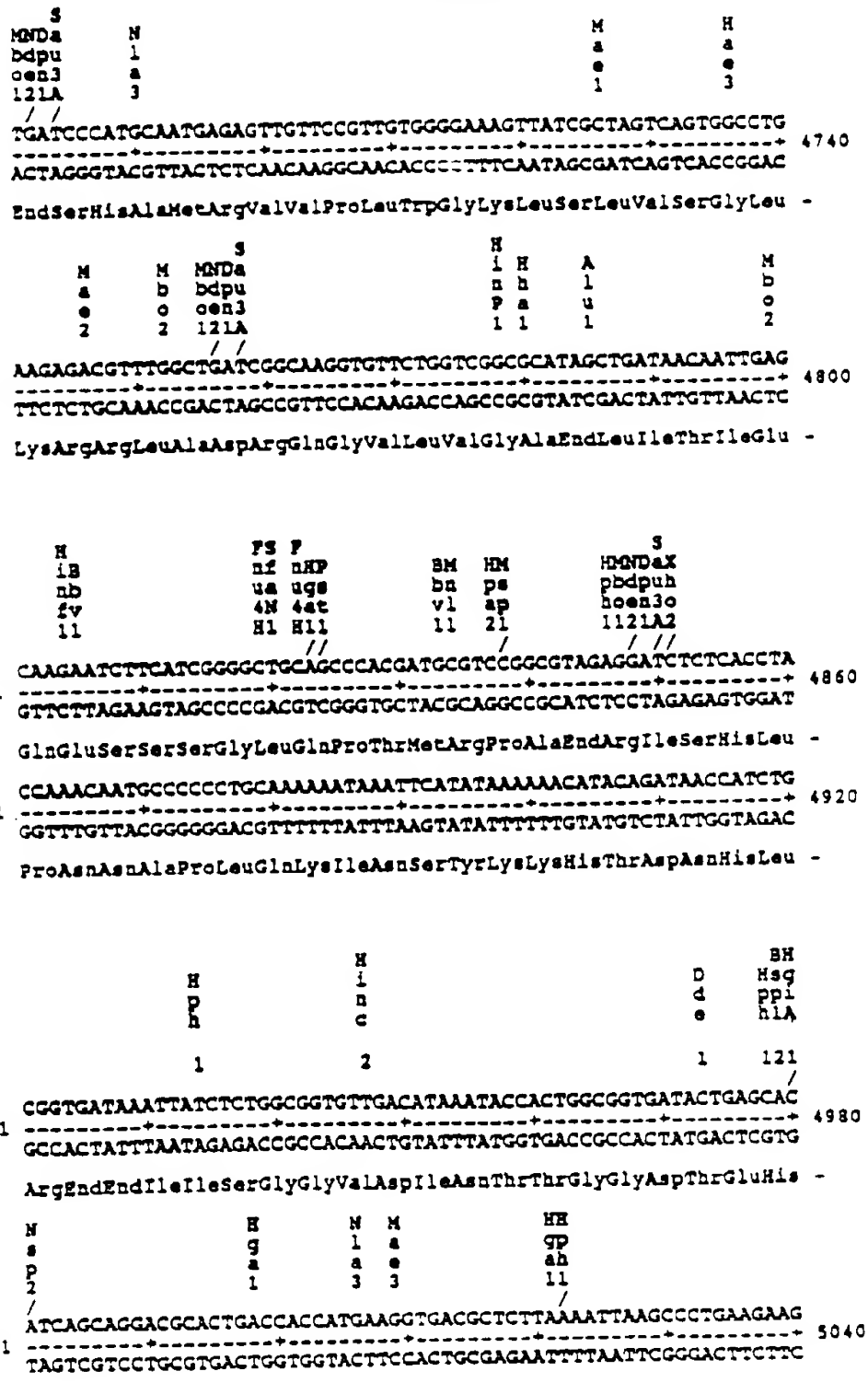


FIG. 11(cont'd)

```
          F
          n BM        B
          u sb        b
          4 mo        v
          H 12        1
         GGCAGCATTCAAAGCAGAAGGCTTTGGGGTGTGTGATACGAAACGAAGCATT
5041     ---------+---------+---------+---------+---------+-- 5092
         CCGTCGTAAGTTTCGTCTTCCGAAACCCCACACACTATGCTTTGCTTCGTAA

         GlySerIleGlnSerArgArgLeuTrpGlyValEndTyrGluThrLysHis??? -
```

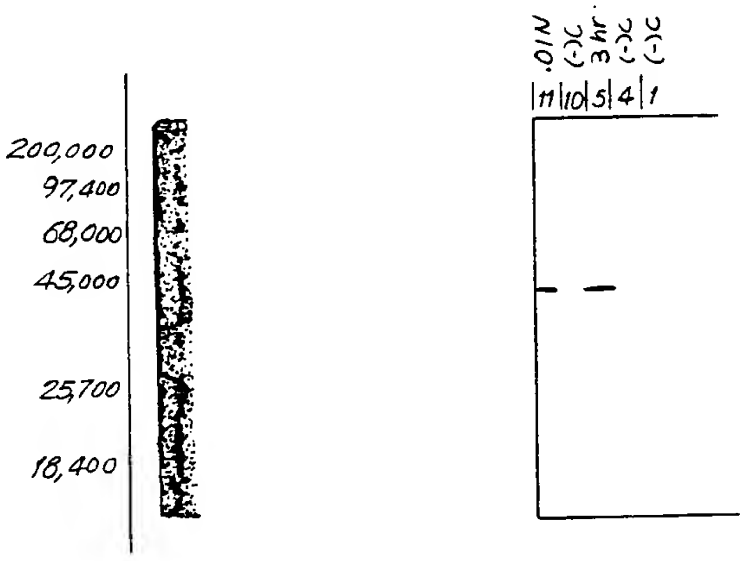
Enzymes that do cut:

Acc1	Aha2	Af12	Alu1	ApaL1	Ava1	Ava2	Ban1	Ban2	Bbe1	Bbv1
Bcl1	Bgl1	Bgl2	Bsm1	Bsp12	BspM1	BstE2	BstN1	BstX1	Cfr1	Cla1
Dde1	Dpn1	Dra2	Eag1	EcoB	EcoK	EcoR1	EcoR2	EcoRV	FnuD2	Fnu4H
Fok1	Fsp1	Hae2	Hae3	Hga1	HgiA1	HgiD1	Hha1	Hinc2	Hind3	Hinf1
HinP1	Hpa2	Hph1	Mae1	Mae2	Mae3	Mbo1	Mbo2	Mn11	Msp1	Mst1
Mst2	Nae1	Nar1	Nci1	Nde1	Nde2	Nhe1	Nla3	Nla4	Nru1	Nsi1
Nsp2	NspB2	NspH1	Pf1M1	PpuM1	Pss1	Pst1	Pvu2	Rsa1	Sac1	Sal1
Sau3A	Sau96	ScrF1	SfaN1	Sin1	Set1	Stu1	Sty1	Taq1	Tha1	Tth1
Xho2	Xma3									

Enzymes that do not cut:

Aat2	Aha3	Apa1	Asp70	Asp71	Asu2	Avr2	Bal1	BamH1	BspM2	Bss$2
Dra1	Dra3	Esp1	Hpa1	Kpn1	Mlu1	Nco1	Not1	PaeR7	Pvu1	Rsr2
Sac2	Sca1	Sfi1	Sma1	SnaB1	Spe1	Sph1	Sse1	Sst2	Xba1	Xho1
Xma1	Xmn1	Xor2								

FIG. 12



lane 1 = Pre-induced
lane 4 = Uninduced
lane 5 = 3 hr. post-induction
lane 11 = overnight post-induction (~16 hr)
MW = Molecular wt. markers

FIG. 13

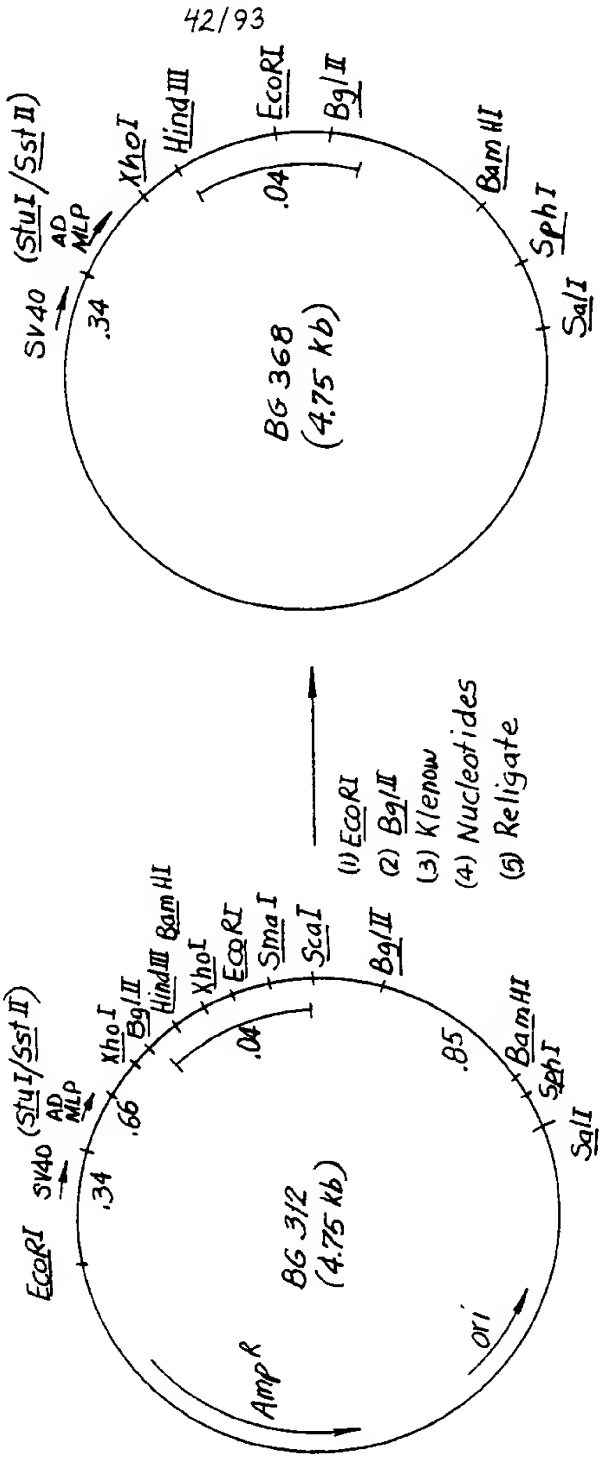

SV40
(StuI/SstII)
AD MLP
XhoI
HindIII
EcoRI
BglII
BamHI
SphI
SalI
.34
.04
BG 368
(4.75 kb)
EcoRI
SV40
(StuI/SstII)
AD MLP
XhoI
BglII
HindIII
BamHI
XhoI
EcoRI
SmaI
ScaI
BglII
BamHI
SphI
SalI
.34
.66
.04
.85
BG 312
(4.75 kb)
Amp^R
ori
(1) EcoRI
(2) BglII
(3) Klenow
(4) Nucleotides
(5) Religate

FIG. 14A

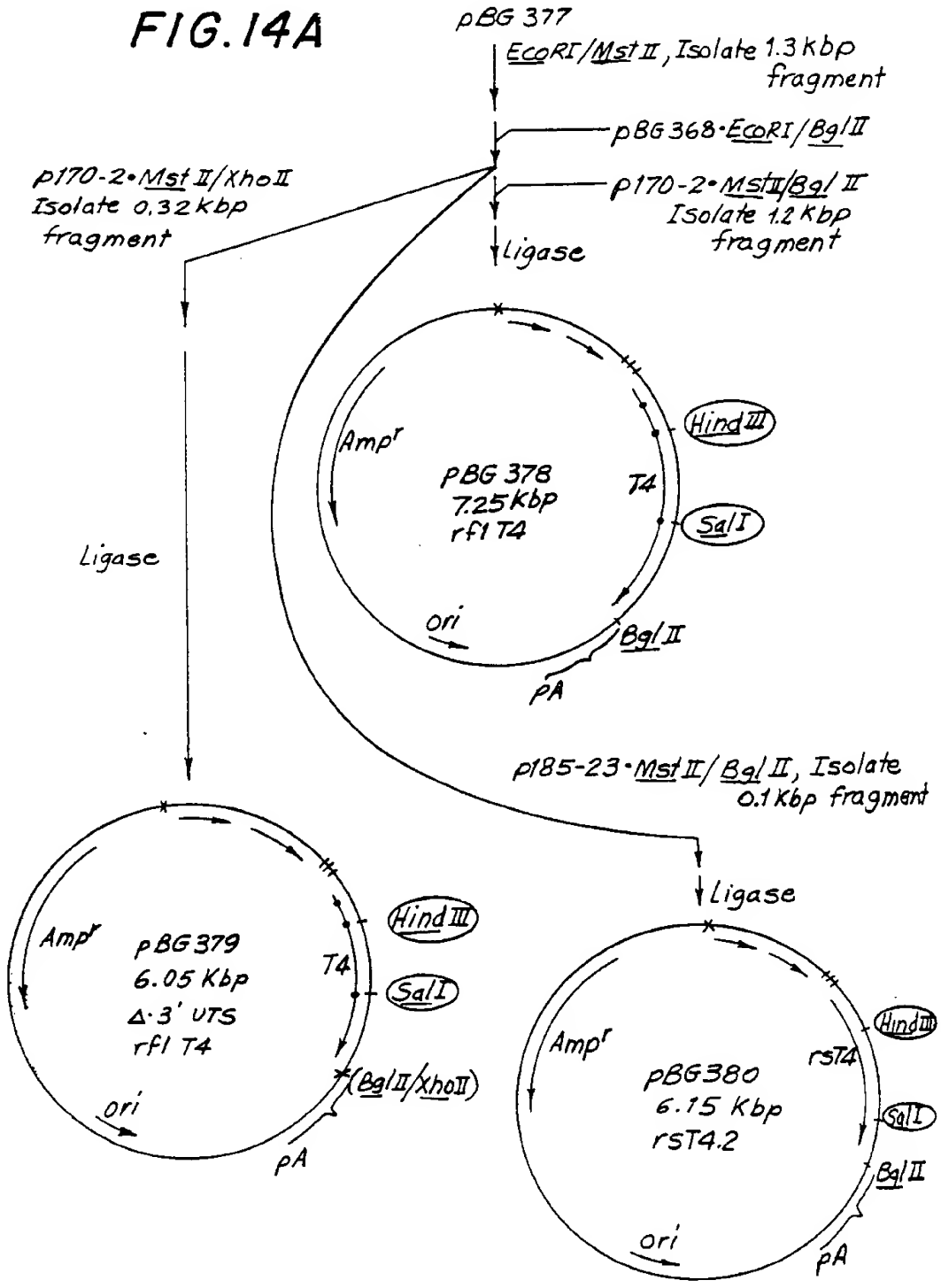

pBG 377
EcoRI/MstII, Isolate 1.3 kbp fragment
pBG 368·EcoRI/BglII
p170-2·MstII/BglII
Isolate 1.2 kbp fragment
Ligase
p170-2·MstII/XhoII
Isolate 0.32 kbp fragment
Ligase
Ampr
pBG 378
7.25 kbp
rf1 T4
T4
HindIII
SalI
ori
BglII
pA
p185-23·MstII/BglII, Isolate 0.1 kbp fragment
Ligase
Ampr
pBG 379
6.05 Kbp
Δ·3' UTS
rf1 T4
T4
HindIII
SalI
ori
(BglII/XhoII)
pA
Ligase
Ampr
pBG380
6.15 Kbp
rsT4.2
rsT4
HindIII
SalI
BglII
ori
pA

FIG. 14B

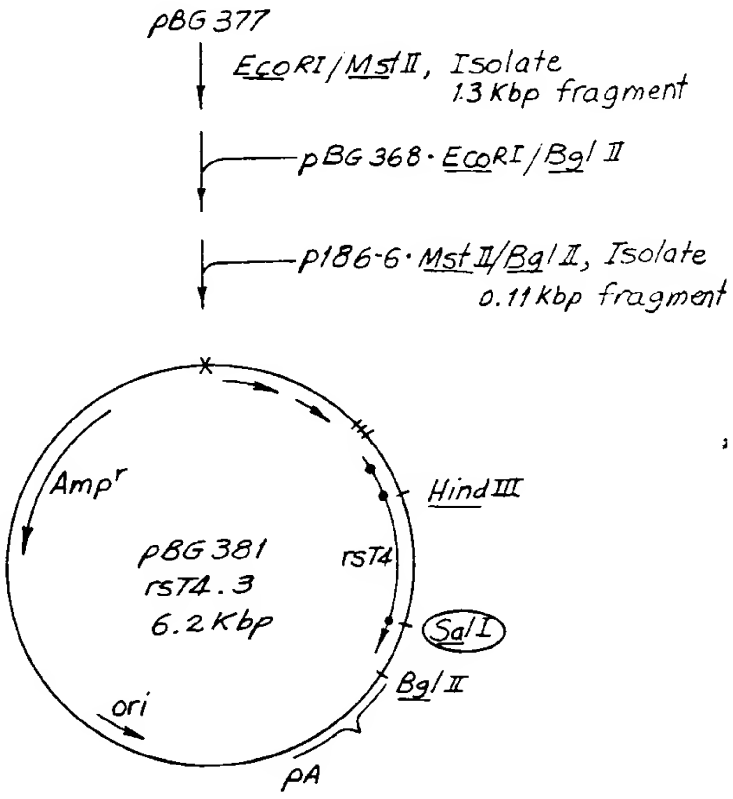

pBG377
EcoRI/MstII, Isolate
1.3 Kbp fragment
pBG368 · EcoRI/BglII
p186-6 · MstII/BglII, Isolate
0.11 kbp fragment
Ampr
HindIII
pBG381
rsT4.3
6.2 Kbp
rsT4
SalI
BglII
ori
pA

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FIG. 14C

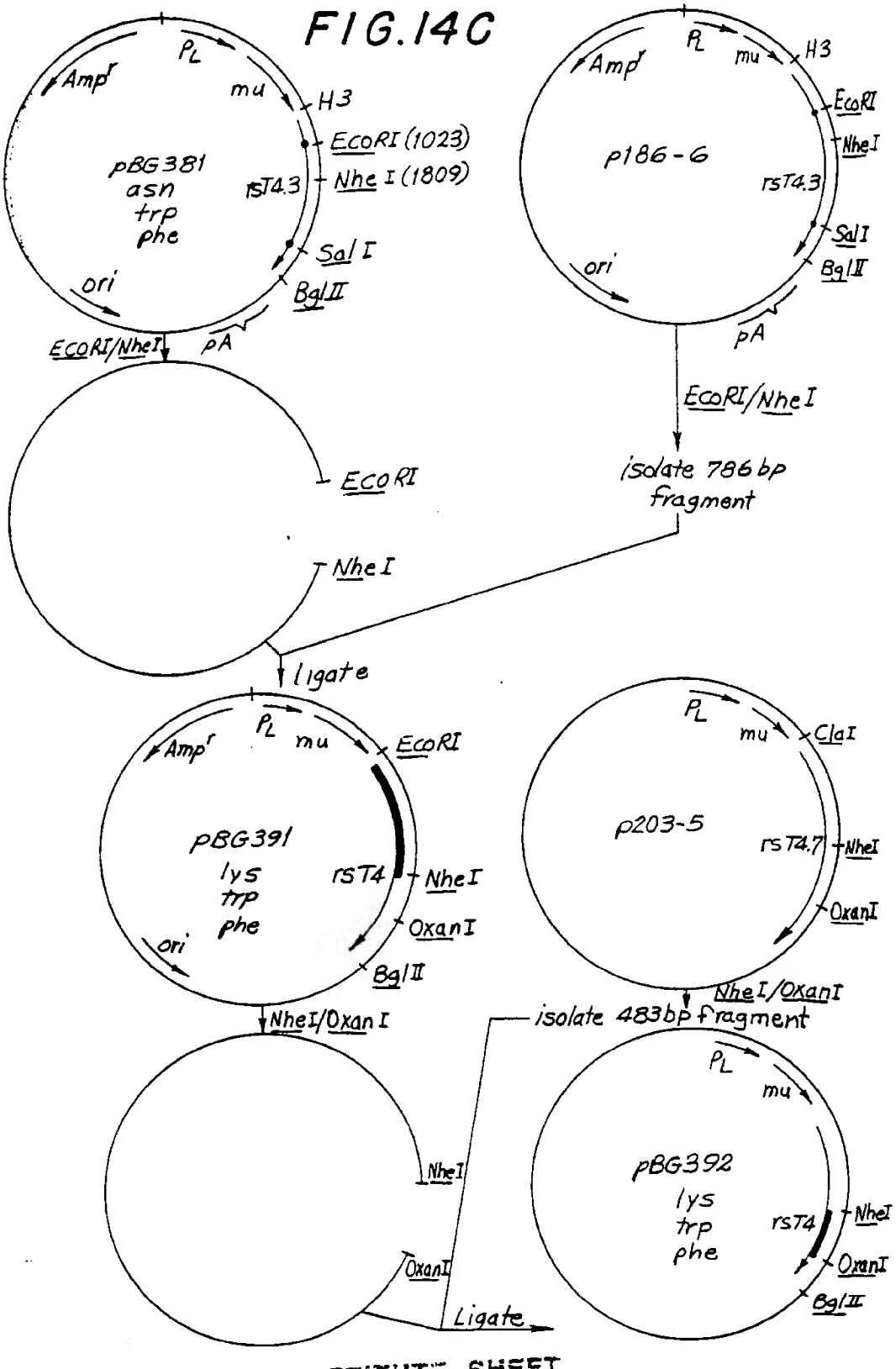

Amp^r
P_L
mu
H3
EcoRI (1023)
Nhe I (1809)
rsT4.3
Sal I
Bgl II
ori
pA
pBG381
asn
trp
phe
EcoRI/NheI
EcoRI
NheI
ligate
p186-6
EcoRI
NheI
SalI
Bgl II
EcoRI/NheI
isolate 786 bp fragment
pBG391
lys
trp
phe
rsT4
OxanI
NheI/OxanI
p203-5
ClaI
rsT4.7
isolate 483bp fragment
pBG392
Ligate

FIG. 15

pBG391
:BG368 backbone
:soluble T4#3
:AA #3 = LYS

bg381.seq Length: 6151

```
   1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
  51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
 101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
 151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
 201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
 251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
 301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
 351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
 401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
 451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
 501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
 551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
 601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
 651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
 701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
 751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
 801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
 851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
 901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
 951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
1401  GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
```

FIG. 15(cont'd)

```
1451  CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501  CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551  GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601  ACCTGCTTCA GGGGCAGAGC CTGACCCTGA CCTTGGAGAG CCCCCCTGGT
1651  AGTAGCCCCT CAGTGCAATG TAGGAGTCCA AGGGGTAAAA ACATACAGGG
1701  GGGGAAGACC CTCTCCGTGT CTCAGCTGGA GCTCCAGGAT AGTGGCACCT
1751  GGACATGCAC TGTCTTGCAG AACCAGAAGA AGGTGGAGTT CAAAATAGAC
1801  ATCGTGGTGC TAGCTTTCCA GAAGGCCTCC AGCATAGTCT ATAAGAAAGA
1851  GGGGGAACAG GTGGAGTTCT CCTTCCCACT CGCCTTTACA GTTGAAAAGC
1901  TGACGGGCAG TGGCGAGCTG TGGTGGCAGG CGGAGAGGGC TTCCTCCTCC
1951  AAGTCTTGGA TCACCTTTGA CCTGAAGAAC AAGGAAGTGT CTGTAAAACG
2001  GGTTACCCAG GACCCTAAGC TCCAGATGGG CAAGAAGCTC CCGCTCCACC
2051  TCACCCTGCC CCAGGCCTTG CCTCAGTATG CTGGCTCTGG AAACCTCACC
2101  CTGGCCCTTG AAGCGAAAAC AGGAAAGTTG CATCAGGAAG TGAACCTGGT
2151  GGTGATGAGA GCCACTCAGC TCCAGAAAAA TTTGACCTGT GAGGTGTGGG
2201  GACCCACCTC CCCTAAGCTG ATGCTGAGTT TGAAACTGGA GAACAAGGAG
2251  GCAAAGGTCT CGAAGCGGGA GAAGGCGGTG TGGGTGCTGA ACCCTGAGGC
2301  GGGGATGTGG CAGTGTCTGC TGAGTGACTC GGGACAGGTC CTGCTGGAAT
2351  CCAACATCAA GGTTCTGCCC ACATGGTCGA CCCCGGTGCA GCCAATGGCC
2401  CTGATTTGAG ATCTTTGTGA AGGAACCTTA CTTCTGTGGT GTGACATAAT
2451  TGGACAAACT ACCTACAGAG ATTTAAAGCT CTAAGGTAAA TATAAAATTT
2501  TTAAGTGTAT AATGTGTTAA ACTACTGATT CTAATTGTTT GTGTATTTTA
2551  GATTCCAACC TATGGAACTG ATGAATGGGA GCAGTGGTGG AATGCCTTTA
2601  ATGAGGAAAA CCTGTTTTGC TCAGAAGAAA TGCCATCTAG TGATGATGAG
2651  GCTACTGCTG ACTCTCAACA TTCTACTCCT CCAAAAAAGA AGAGAAAGGT
2701  AGAAGACCCC AAGGACTTTC CTTCAGAATT GCTAAGTTTT TTGAGTCATG
2751  CTGTGTTTAG TAATAGAACT CTTGCTTGCT TTGCTATTTA CACCACAAAG
2801  GAAAAAGCTG CACTGCTATA CAAGAAAATT ATGGAAAAAT ATTCTGTAAC
2851  CTTTATAAGT AGGCATAACA GTTATAATCA TAACATACTG TTTTTTCTTA
2901  CTCCACACAG GCATAGAGTG TCTGCTATTA ATAACTATGC TCAAAAATTG
2951  TGTACCTTTA GCTTTTTAAT TTGTAAAGGG GTTAATAAGG AATATTTGAT
3001  GTATAGTGCC TTGACTAGAG ATCATAATCA GCCATACCAC ATTTGTAGAG
3051  GTTTTACTTG CTTTAAAAAA CCTCCCACAC CTCCCCCTGA ACCTGAAACA
```

FIG. 15(cont'd)

```
3101  TAAAATGAAT GCAATTGTTG TTGTTAACTT GTTTATTGCA GCTTATAATG
3151  GTTACAAATA AAGCAATAGC ATCACAAATT TCACAAATAA AGCATTTTTT
3201  TCACTGCATT CTAGTTGTGG TTTGTCCAAA CTCATCAATG TATCTTATCA
3251  TGTCTGGATC CTCTACGCCG GACGCATCGT GGCCGGCATC ACCGGCGCCA
3301  CAGGTGCGGT TGCTGGCGCC TATATCGCCG ACATCACCGA TGGGGAAGAT
3351  CGGGCTCGCC ACTTCGGGCT CATGAGCGCT TGTTTCGGCG TGGGTATGGT
3401  GGCAGGCCCG TGGCCGGGGG ACTGTTGGGC GCCATCTCCT TGCATGCACC
3451  ATTCCTTGCG GCGGCGGTGC TCAACGGCCT CAACCTACTA CTGGGCTGCT
3501  TCCTAATGCA GGAGTCGCAT AAGGGAGAGC GTCGACCGAT GCCCTTGAGA
3551  GCCTTCAACC CAGTCAGCTC CTTCCGGTGG GCGCGGGGCA TGACTATCGT
3601  CGCCGCACTT ATGACTGTCT TCTTTATCAT GCAACTCGTA GGACAGGTGC
3651  CGGCAGCGCT CTGGGTCATT TTCGGCGAGG ACCGCTTTCG CTGGAGCGCG
3701  ACGATGATCG GCCTGTCGCT TGCGGTATTC GGAATCTTGC ACGCCCTCGC
3751  TCAAGCCTTC GTCACTGGTC CCGCCACCAA ACGTTTCGGC GAGAAGCAGG
3801  CCATTATCGC CGGCATGGCG GCCGACGCGC TGGGCTACGT CTTGCTGGCG
3851  TTCGCGACGC GAGGCTGGAT GGCCTTCCCC ATTATGATTC TTCTCGCTTC
3901  CGGCGGCATC GGGATGCCCG CGTTGCAGGC CATGCTGTCC AGGCAGGTAG
3951  ATGACGACCA TCAGGGACAG CTTCAAGGAT CGCTCGCGGC TCTTACCAGC
4001  CTAACTTCGA TCACTGGACC GCTGATCGTC ACGGCGATTT ATGCCGCCTC
4051  GGCGAGCACA TGGAACGGGT TGGCATGGAT TGTAGGCGCC GCCCTATACC
4101  TTGTCTGCCT CCCCGCGTTG CGTCGCGGTG CATGGAGCCG GGCCACCTCG
4151  ACCTGAATGG AAGCCGGCGG CACCTCGCTA ACGGATTCAC CACTCCAAGA
4201  ATTGGAGCCA ATCAATTCTT GCGGAGAACT GTGAATGCGC AAACCAACCC
4251  TTGGCAGAAC ATATCCATCG CGTCCGCCAT CTCCAGCAGC CGCACGCGGC
4301  GCATCTCGGG CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA
4351  CGAGCATCAC AAAAATCGAC GCTCAAGTCA GAGGTGGCGA AACCCGACAG
4401  GACTATAAAG ATACCAGGCG TTTCCCCCTG GAAGCTCCCT CGTGCGCTCT
4451  CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT TTCTCCCTTC
4501  GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG
4551  TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG
4601  CCCGACCGCT GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT
4651  AAGACACGAC TTATCGCCAC TGGCAGCAGC CACTGGTAAC AGGATTAGCA
4701  GAGCGAGGTA TGTAGGCGGT GCTACAGAGT TCTTGAAGTG GTGGCCTAAC
```

FIG. 15(cont'd)

```
4751  TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC TGCTGAAGCC
4801  AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA
4851  CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA
4901  AAAAAAGGAT CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC
4951  TCAGTGGAAC GAAAACTCAC GTTAAGGGAT TTTGGTCATG AGATTATCAA
5001  AAAGGATCTT CACCTAGATC CTTTTAAATT AAAAATGAAG TTTTAAATCA
5051  ATCTAAAGTA TATATGAGTA AACTTGGTCT GACAGTTACC AATGCTTAAT
5101  CAGTGAGGCA CCTATCTCAG CGATCTGTCT ATTTCGTTCA TCCATAGTTG
5151  CCTGACTCCC CGTCGTGTAG ATAACTACGA TACGGGAGGG CTTACCATCT
5201  GGCCCCAGTG CTGCAATGAT ACCGCGAGAC CCACGCTCAC CGGCTCCAGA
5251  TTTATCAGCA ATAAACCAGC CAGCCGGAAG GGCCGAGCGC AGAAGTGGTC
5301  CTGCAACTTT ATCCGCCTCC ATCCAGTCTA TTAATTGTTG CCGGGAAGCT
5351  AGAGTAAGTA GTTCGCCAGT TAATAGTTTG CGCAACGTTG TTGCCATTGC
5401  TGCAGGCATC GTGGTGTCAC GCTCGTCGTT TGGTATGGCT TCATTCAGCT
5451  CCGGTTCCCA ACGATCAAGG CGAGTTACAT GATCCCCCAT GTTGTGCAAA
5501  AAAGCGGTTA GCTCCTTCGG TCCTCCGATC GTTGTCAGAA GTAAGTTGGC
5551  CGCAGTGTTA TCACTCATGG TTATGGCAGC ACTGCATAAT TCTCTTACTG
5601  TCATGCCATC CGTAAGATGC TTTTCTGTGA CTGGTGAGTA CTCAACCAAG
5651  TCATTCTGAG AATAGTGTAT GCGGCGACCG AGTTGCTCTT GCCCGGCGTC
5701  AACACGGGAT AATACCGCGC CACATAGCAG AACTTTAAAA GTGCTCATCA
5751  TTGGAAAACG TTCTTCGGGG CGAAAACTCT CAAGGATCTT ACCGCTGTTG
5801  AGATCCAGTT CGATGTAACC CACTCGTGCA CCCAACTGAT CTTCAGCATC
5851  TTTTACTTTC ACCAGCGTTT CTGGGTGAGC AAAAACAGGA AGGCAAAATG
5901  CCGCAAAAAA GGGAATAAGG GCGACACGGA AATGTTGAAT ACTCATACTC
5951  TTCCTTTTTC AATATTATTG AAGCATTTAT CAGGGTTATT GTCTCATGAG
6001  CGGATACATA TTTGAATGTA TTTAGAAAAA TAAACAAATA GGGGTTCCGC
6051  GCACATTTCC CCGAAAAGTG CCACCTGACG TCTAAGAAAC CATTATTATC
6101  ATGACATTAA CCTATAAAAA TAGGCGTATC ACGAGGCCCT TTCGTCTTCA
6151  A
```

FIG. 16

```
pBG392
:BG368 backbone
:soluble T4#7
:AA #3 = LYS
:182AA+6AA
:From 203-5

      bg392.seq  Length: 6149

     1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
    51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
   101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
   151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
   201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
   251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
   301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
   351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
   401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
   451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
   501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
   551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
   601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
   651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
   701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
   751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
   801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
   851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
   901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
   951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
  1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
  1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
  1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
  1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
                MET
  1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
          ←AA-23
  1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
                          ←AA1
  1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
  1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
```

FIG. 16(cont'd)

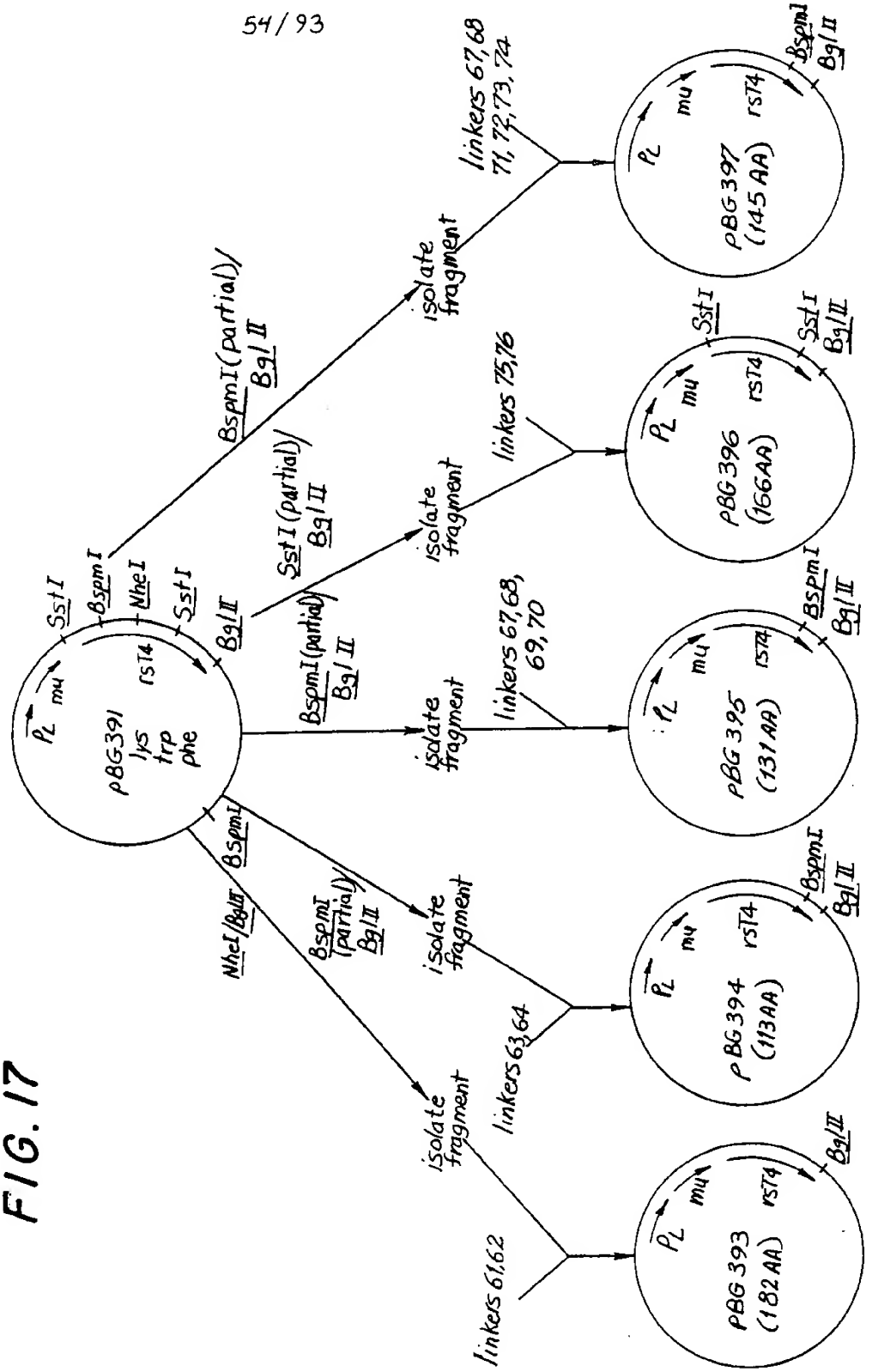
```
1401  GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
1451  CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501  CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551  GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601  ACCTGCTTCA GGGGCAGAGC CTGACCCTGA CCTTGGAGAG CCCCCCTGGT
1651  AGTAGCCCCT CAGTGCAATG TAGGAGTCCA AGGGGTAAAA ACATACAGGG
1701  GGGGAAGACC CTCTCCGTGT CTCAGCTGGA GCTCCAGGAT AGTGGCACCT
1751  GGACATGCAC TGTCTTGCAG AACCAGAAGA AGGTGGAGTT CAAAATAGAC
                                          STOP
1801  ATCGTGGTGC TAGCTTTCCA GAACCTCCAG CATAGTCTAT AAGAAAGAGG
1851  GGGAACAGGT GGAGTTCTCC TTCCCACTCG CCTTTACAGT TGAAAAGCTG
1901  ACGGGCAGTG GCGAGCTGTG GTGGCAGGCG GAGAGGGCTT CCTCCTCCAA
1951  GTCTTGGATC ACCTTTGACC TGAAGAACAA GGAAGTGTCT GTAAAACGGG
2001  TTACCCAGGA CCCTAAGCTC CAGATGGGCA AGAAGCTCCC GCTCCACCTC
2051  ACCCTGCCCC AGGCCTTGCC TCAGTATGCT GGCTCTGGAA ACCTCACCCT
2101  GGCCCTTGAA GCGAAAACAG GAAAGTTGCA TCAGGAAGTG AACCTGGTGG
2151  TGATGAGAGC CACTCAGCTC CAGAAAAATT TGACCTGTGA GGTGTGGGGA
2201  CCCACCTCCC CTAAGCTGAT GCTGAGTTTG AAACTGGAGA ACAAGGAGGC
2251  AAAGGTCTCG AAGCGGGAGA AGGCGGTGTG GGTGCTGAAC CCTGAGGCGG
2301  GGATGTGGCA GTGTCTGCTG AGTGACTCGG GACAGGTCCT GCTGGAATCC
2351  AACATCAAGG TTCTGCCCAC ATGGTCGACC CCGGTGCAGC CAATGGCCCT
2401  GATTTGAGAT CTTTGTGAAG GAACCTTACT TCTGTGGTGT GACATAATTG
2451  GACAAACTAC CTACAGAGAT TTAAAGCTCT AAGGTAAATA TAAAATTTTT
2501  AAGTGTATAA TGTGTTAAAC TACTGATTCT AATTGTTTGT GTATTTTAGA
2551  TTCCAACCTA TGGAACTGAT GAATGGGAGC AGTGGTGGAA TGCCTTTAAT
2601  GAGGAAAACC TGTTTTGCTC AGAAGAAATG CCATCTAGTG ATGATGAGGC
2651  TACTGCTGAC TCTCAACATT CTACTCCTCC AAAAAAGAAG AGAAAGGTAG
2701  AAGACCCCAA GGACTTTCCT TCAGAATTGC TAAGTTTTTT GAGTCATGCT
2751  GTGTTTAGTA ATAGAACTCT TGCTTGCTTT GCTATTTACA CCACAAAGGA
2801  AAAAGCTGCA CTGCTATACA AGAAAATTAT GGAAAAATAT TCTGTAACCT
2851  TTATAAGTAG GCATAACAGT TATAATCATA ACATACTGTT TTTTCTTACT
2901  CCACACAGGC ATAGAGTGTC TGCTATTAAT AACTATGCTC AAAAATTGTG
2951  TACCTTTAGC TTTTTAATTT GTAAAGGGGT TAATAAGGAA TATTTGATGT
3001  ATAGTGCCTT GACTAGAGAT CATAATCAGC CATACCACAT TTGTAGAGGT
```

FIG. 16(cont'd)

```
3051  TTTACTTGCT TTAAAAAACC TCCCACACCT CCCCCTGAAC CTGAAACATA
3101  AAATGAATGC AATTGTTGTT GTTAACTTGT TTATTGCAGC TTATAATGGT
3151  TACAAATAAA GCAATAGCAT CACAAATTTC ACAAATAAAG CATTTTTTTC
3201  ACTGCATTCT AGTTGTGGTT TGTCCAAACT CATCAATGTA TCTTATCATG
3251  TCTGGATCCT CTACGCCGGA CGCATCGTGG CCGGCATCAC CGGCGCCACA
3301  GGTGCGGTTG CTGGCGCCTA TATCGCCGAC ATCACCGATG GGGAAGATCG
3351  GGCTCGCCAC TTCGGGCTCA TGAGCGCTTG TTTCGGCGTG GGTATGGTGG
3401  CAGGCCCGTG GCCGGGGGAC TGTTGGGCGC CATCTCCTTG CATGCACCAT
3451  TCCTTGCGGC GGCGGTGCTC AACGGCCTCA ACCTACTACT GGGCTGCTTC
3501  CTAATGCAGG AGTCGCATAA GGGAGAGCGT CGACCGATGC CCTTGAGAGC
3551  CTTCAACCCA GTCAGCTCCT TCCGGTGGGC GCGGGGCATG ACTATCGTCG
3601  CCGCACTTAT GACTGTCTTC TTTATCATGC AACTCGTAGG ACAGGTGCCG
3651  GCAGCGCTCT GGGTCATTTT CGGCGAGGAC CGCTTTCGCT GGAGCGCGAC
3701  GATGATCGGC CTGTCGCTTG CGGTATTCGG AATCTTGCAC GCCCTCGCTC
3751  AAGCCTTCGT CACTGGTCCC GCCACCAAAC GTTTCGGCGA GAAGCAGGCC
3801  ATTATCGCCG GCATGGCGGC CGACGCGCTG GGCTACGTCT TGCTGGCGTT
3851  CGCGACGCGA GGCTGGATGG CCTTCCCCAT TATGATTCTT CTCGCTTCCG
3901  GCGGCATCGG GATGCCCGCG TTGCAGGCCA TGCTGTCCAG GCAGGTAGAT
3951  GACGACCATC AGGGACAGCT TCAAGGATCG CTCGCGGCTC TTACCAGCCT
4001  AACTTCGATC ACTGGACCGC TGATCGTCAC GGCGATTTAT GCCGCCTCGG
4051  CGAGCACATG GAACGGGTTG GCATGGATTG TAGGCGCCGC CCTATACCTT
4101  GTCTGCCTCC CCGCGTTGCG TCGCGGTGCA TGGAGCCGGG CCACCTCGAC
4151  CTGAATGGAA GCCGGCGGCA CCTCGCTAAC GGATTCACCA CTCCAAGAAT
4201  TGGAGCCAAT CAATTCTTGC GGAGAACTGT GAATGCGCAA ACCAACCCTT
4251  GGCAGAACAT ATCCATCGCG TCCGCCATCT CCAGCAGCCG CACGCGGCGC
4301  ATCTCGGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG
4351  AGCATCACAA AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA
4401  CTATAAAGAT ACCAGGCGTT TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC
4451  TGTTCCGACC CTGCCGCTTA CCGGATACCT GTCCGCCTTT CTCCCTTCGG
4501  GAAGCGTGGC GCTTTCTCAA TGCTCACGCT GTAGGTATCT CAGTTCGGTG
4551  TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCC CCGTTCAGCC
4601  CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA
4651  GACACGACTT ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA
```

FIG. 16(cont'd)

```
4701  GCGAGGTATG TAGGCGGTGC TACAGAGTTC TTGAAGTGGT GGCCTAACTA
4751  CGGCTACACT AGAAGGACAG TATTTGGTAT CTGCGCTCTG CTGAAGCCAG
4801  TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA ACAAACCACC
4851  GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA CGCGCAGAAA
4901  AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC
4951  AGTGGAACGA AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA
5001  AGGATCTTCA CCTAGATCCT TTTAAATTAA AAATGAAGTT TTAAATCAAT
5051  CTAAAGTATA TATGAGTAAA CTTGGTCTGA CAGTTACCAA TGCTTAATCA
5101  GTGAGGCACC TATCTCAGCG ATCTGTCTAT TTCGTTCATC CATAGTTGCC
5151  TGACTCCCCG TCGTGTAGAT AACTACGATA CGGGAGGGCT TACCATCTGG
5201  CCCCAGTGCT GCAATGATAC CGCGAGACCC ACGCTCACCG GCTCCAGATT
5251  TATCAGCAAT AAACCAGCCA GCCGGAAGGG CCGAGCGCAG AAGTGGTCCT
5301  GCAACTTTAT CCGCCTCCAT CCAGTCTATT AATTGTTGCC GGGAAGCTAG
5351  AGTAAGTAGT TCGCCAGTTA ATAGTTTGCG CAACGTTGTT GCCATTGCTG
5401  CAGGCATCGT GGTGTCACGC TCGTCGTTTG GTATGGCTTC ATTCAGCTCC
5451  GGTTCCCAAC GATCAAGGCG AGTTACATGA TCCCCCATGT TGTGCAAAAA
5501  AGCGGTTAGC TCCTTCGGTC CTCCGATCGT TGTCAGAAGT AAGTTGGCCG
5551  CAGTGTTATC ACTCATGGTT ATGGCAGCAC TGCATAATTC TCTTACTGTC
5601  ATGCCATCCG TAAGATGCTT TTCTGTGACT GGTGAGTACT CAACCAAGTC
5651  ATTCTGAGAA TAGTGTATGC GGCGACCGAG TTGCTCTTGC CCGGCGTCAA
5701  CACGGGATAA TACCGCGCCA CATAGCAGAA CTTTAAAAGT GCTCATCATT
5751  GGAAAACGTT CTTCGGGGCG AAAACTCTCA AGGATCTTAC CGCTGTTGAG
5801  ATCCAGTTCG ATGTAACCCA CTCGTGCACC CAACTGATCT TCAGCATCTT
5851  TTACTTTCAC CAGCGTTTCT GGGTGAGCAA AAACAGGAAG GCAAAATGCC
5901  GCAAAAAAGG GAATAAGGGC GACACGGAAA TGTTGAATAC TCATACTCTT
5951  CCTTTTTCAA TATTATTGAA GCATTTATCA GGGTTATTGT CTCATGAGCG
6001  GATACATATT TGAATGTATT TAGAAAAATA AACAAATAGG GGTTCCGCGC
6051  ACATTTCCCC GAAAAGTGCC ACCTGACGTC TAAGAAACCA TTATTATCAT
6101  GACATTAACC TATAAAAATA GGCGTATCAC GAGGCCCTTT CGTCTTCAA
```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trp
phe
pL
mu
rst4
Sst I
BspmI
NheI
Sst I
BglII
NheI/BglII
BspmI
BspmI (partial)/ BglII
Sst I (partial)/ BglII
BspmI (partial)/ BglII
isolate fragment
linkers 61,62
linkers 63,64
linkers 67,68, 69,70
linkers 75,76
linkers 67,68 71,72,73,74
pBG393 (182 AA)
pBG394 (113 AA)
pBG395 (131 AA)
pBG396 (166 AA)
pBG397 (145 AA)
BspmI
BglII
Sst I

FIG. 17

FIG. 18

61
5' CTA GCT TTT CCA GTG A 3'

62
5' GAT CTC ACT GGA AAG 3'

63
5' GGG GTG ATA GTA A 3'

64
5' GAT CTT ACT ATC A 3'

67
5' GGG GCA GAG CCT GAC CCT GAC CTT GGA GAG CCC C 3'

68
5' CCC GGG GGC TCT CCA AGG TCA GGG TCA GGC TCT G 3'

69
5' CCG GGT AGT AGC CCC TCA GTG CAA TGA 3'

70
5' GAT CTC ATT GCA CTG AGG GGC TAC TAC 3'

71
5' CCG GGT AGT AGC CCC TCA GTG CAA TGT AGG AGT C 3'

72
5' TAG GAC TCC TAC ATT GCA CTG AGG GGC TAC TAC 3'

73
5' CTA GGG GTA AAA ACA TAC AGG GGG GGA AGA CCT GA 3'

74
5' GAT CTC AGG TCT TTC CCC CCC TGT ATG TTT TTA CCC 3'

75
5' CCA GGA TAG TGG CAC CTG GAC ATG CAC TGT CTT GCA
GAA CTG A 3'

76
5' GAT CTC AGT TCT GCA AGA CAG TGC ATG TCC AGG TGC
CAC TAT CCT GGA GCT 3'

FIG. 19

pBG394
:BG368 backbone
:soluble T4#9
:AA #3 = LYS
:first 113 AA of T4
:basically up to V1J1

bg394.seq Length: 5365

```
   1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
  51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
 101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
 151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
 201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
 251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
 301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
 351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
 401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
 451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
 501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
 551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
 601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
 651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
 701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
 751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
 801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
 851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
 901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
 951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
```

FIG. 19 (cont'd)

1401 GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
1451 CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501 CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551 GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601 ACCTGCTTCA GGGGTGATAG TAAGATCTTT GTGAAGGAAC CTTACTTCTG
1651 TGGTGTGACA TAATTGGACA AACTACCTAC AGAGATTTAA AGCTCTAAGG
1701 TAAATATAAA ATTTTTAAGT GTATAATGTG TTAAACTACT GATTCTAATT
1751 GTTTGTGTAT TTTAGATTCC AACCTATGGA ACTGATGAAT GGGAGCAGTG
1801 GTGGAATGCC TTTAATGAGG AAAACCTGTT TTGCTCAGAA GAAATGCCAT
1851 CTAGTGATGA TGAGGCTACT GCTGACTCTC AACATTCTAC TCCTCCAAAA
1901 AAGAAGAGAA AGGTAGAAGA CCCCAAGGAC TTTCCTTCAG AATTGCTAAG
1951 TTTTTTGAGT CATGCTGTGT TTAGTAATAG AACTCTTGCT TGCTTTGCTA
2001 TTTACACCAC AAAGGAAAAA GCTGCACTGC TATACAAGAA AATTATGGAA
2051 AAATATTCTG TAACCTTTAT AAGTAGGCAT AACAGTTATA ATCATAACAT
2101 ACTGTTTTTT CTTACTCCAC ACAGGCATAG AGTGTCTGCT ATTAATAACT
2151 ATGCTCAAAA ATTGTGTACC TTTAGCTTTT TAATTTGTAA AGGGGTTAAT
2201 AAGGAATATT TGATGTATAG TGCCTTGACT AGAGATCATA ATCAGCCATA
2251 CCACATTTGT AGAGGTTTTA CTTGCTTTAA AAAACCTCCC ACACCTCCCC
2301 CTGAACCTGA AACATAAAAT GAATGCAATT GTTGTTGTTA ACTTGTTTAT
2351 TGCAGCTTAT AATGGTTACA AATAAAGCAA TAGCATCACA AATTTCACAA
2401 ATAAAGCATT TTTTTCACTG CATTCTAGTT GTGGTTTGTC CAAACTCATC
2451 AATGTATCTT ATCATGTCTG GATCCTCTAC GCCGGACGCA TCGTGGCCGG
2501 CATCACCGGC GCCACAGGTG CGGTTGCTGG CGCCTATATC GCCGACATCA
2551 CCGATGGGGA AGATCGGGCT CGCCACTTCG GGCTCATGAG CGCTTGTTTC
2601 GGCGTGGGTA TGGTGGCAGG CCCGTGGCCG GGGGACTGTT GGGCGCCATC
2651 TCCTTGCATG CACCATTCCT TGCGGCGGCG GTGCTCAACG GCCTCAACCT
2701 ACTACTGGGC TGCTTCCTAA TGCAGGAGTC GCATAAGGGA GAGCGTCGAC
2751 CGATGCCCTT GAGAGCCTTC AACCCAGTCA GCTCCTTCCG GTGGGCGCGG
2801 GGCATGACTA TCGTCGCCGC ACTTATGACT GTCTTCTTTA TCATGCAACT
2851 CGTAGGACAG GTGCCGGCAG CGCTCTGGGT CATTTTCGGC GAGGACCGCT
2901 TTCGCTGGAG CGCGACGATG ATCGGCCTGT CGCTTGCGGT ATTCGGAATC
2951 TTGCACGCCC TCGCTCAAGC CTTCGTCACT GGTCCCGCCA CCAAACGTTT
3001 CGGCGAGAAG CAGGCCATTA TCGCCGGCAT GGCGGCCGAC GCGCTGGGCT

FIG. 19 (cont'd)

```
3051  ACGTCTTGCT GGCGTTCGCG ACGCGAGGCT GGATGGCCTT CCCCATTATG
3101  ATTCTTCTCG CTTCCGGCGG CATCGGGATG CCCGCGTTGC AGGCCATGCT
3151  GTCCAGGCAG GTAGATGACG ACCATCAGGG ACAGCTTCAA GGATCGCTCG
3201  CGGCTCTTAC CAGCCTAACT TCGATCACTG GACCGCTGAT CGTCACGGCG
3251  ATTTATGCCG CCTCGGCGAG CACATGGAAC GGGTTGGCAT GGATTGTAGG
3301  CGCCGCCCTA TACCTTGTCT GCCTCCCCGC GTTGCGTCGC GGTGCATGGA
3351  GCCGGGCCAC CTCGACCTGA ATGGAAGCCG GCGGCACCTC GCTAACGGAT
3401  TCACCACTCC AAGAATTGGA GCCAATCAAT TCTTGCGGAG AACTGTGAAT
3451  GCGCAAACCA ACCCTTGGCA GAACATATCC ATCGCGTCCG CCATCTCCAG
3501  CAGCCGCACG CGGCGCATCT CGGGCCGCGT TGCTGGCGTT TTTCCATAGG
3551  CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG
3601  GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT
3651  CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC
3701  GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCAATGCT CACGCTGTAG
3751  GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG
3801  AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT
3851  GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG
3901  TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA
3951  AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC
4001  GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC
4051  CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC
4101  AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT
4151  ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT
4201  CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT
4251  GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT
4301  TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG
4351  TTCATCCATA GTTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG
4401  AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC
4451  TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA
4501  GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT
4551  GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC
4601  GTTGTTGCCA TTGCTGCAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT
4651  GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC
```

FIG. 19 (cont'd)

```
4701  CCATGTTGTG CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC
4751  AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA
4801  TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG
4851  AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC
4901  TCTTGCCCGG CGTCAACACG GGATAATACC GCGCCACATA GCAGAACTTT
4951  AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA
5001  TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC
5051  TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC
5101  AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT
5151  GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT
5201  TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA
5251  AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG
5301  AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG
5351  CCCTTTCGTC TTCAA
```

FIG. 20

pBG396
:BG368 backbone
:soluble T4#12
:AA #3 = LYS

bg396.seq Length: 5518

```
   1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
  51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
 101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
 151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
 201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
 251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
 301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
 351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
 401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
 451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
 501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
 551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
 601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
 651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
 701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
 751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
 801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
 851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
 901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
 951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
1401  GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
```

FIG. 20 (cont'd)

```
1451  CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501  CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551  GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601  ACCTGCTTCA GGGGCAGAGC CTGACCCTGA CCTTGGAGAG CCCCCCTGGT
1651  AGTAGCCCCT CAGTGCAATG TAGGAGTCCA AGGGGTAAAA ACATACAGGG
1701  GGGGAAGACC CTCTCCGTGT CTCAGCTGGA GCTCCAGGAT AGTGGCACCT
1751  GGACATGCAC TGTCTTGCAG AACTGAGATC TTTGTGAAGG AACCTTACTT
1801  CTGTGGTGTG ACATAATTGG ACAAACTACC TACAGAGATT TAAAGCTCTA
1851  AGGTAAATAT AAAATTTTTA AGTGTATAAT GTGTTAAACT ACTGATTCTA
1901  ATTGTTTGTG TATTTTAGAT TCCAACCTAT GGAACTGATG AATGGGAGCA
1951  GTGGTGGAAT GCCTTTAATG AGGAAAACCT GTTTTGCTCA GAAGAAATGC
2001  CATCTAGTGA TGATGAGGCT ACTGCTGACT CTCAACATTC TACTCCTCCA
2051  AAAAAGAAGA GAAAGGTAGA AGACCCCAAG GACTTTCCTT CAGAATTGCT
2101  AAGTTTTTTG AGTCATGCTG TGTTTAGTAA TAGAACTCTT GCTTGCTTTG
2151  CTATTTACAC CACAAAGGAA AAAGCTGCAC TGCTATACAA GAAAATTATG
2201  GAAAAATATT CTGTAACCTT TATAAGTAGG CATAACAGTT ATAATCATAA
2251  CATACTGTTT TTTCTTACTC CACACAGGCA TAGAGTGTCT GCTATTAATA
2301  ACTATGCTCA AAAATTGTGT ACCTTTAGCT TTTTAATTTG TAAAGGGGTT
2351  AATAAGGAAT ATTTGATGTA TAGTGCCTTG ACTAGAGATC ATAATCAGCC
2401  ATACCACATT TGTAGAGGTT TTACTTGCTT TAAAAAACCT CCCACACCTC
2451  CCCCTGAACC TGAAACATAA AATGAATGCA ATTGTTGTTG TTAACTTGTT
2501  TATTGCAGCT TATAATGGTT ACAAATAAAG CAATAGCATC ACAAATTTCA
2551  CAAATAAAGC ATTTTTTTCA CTGCATTCTA GTTGTGGTTT GTCCAAACTC
2601  ATCAATGTAT CTTATCATGT CTGGATCCTC TACGCCGGAC GCATCGTGGC
2651  CGGCATCACC GGCGCCACAG GTGCGGTTGC TGGCGCCTAT ATCGCCGACA
2701  TCACCGATGG GGAAGATCGG GCTCGCCACT TCGGGCTCAT GAGCGCTTGT
2751  TTCGGCGTGG GTATGGTGGC AGGCCCGTGG CCGGGGGACT GTTGGGCGCC
2801  ATCTCCTTGC ATGCACCATT CCTTGCGGCG GCGGTGCTCA ACGGCCTCAA
2851  CCTACTACTG GGCTGCTTCC TAATGCAGGA GTCGCATAAG GGAGAGCGTC
2901  GACCGATGCC CTTGAGAGCC TTCAACCCAG TCAGCTCCTT CCGGTGGGCG
2951  CGGGGCATGA CTATCGTCGC CGCACTTATG ACTGTCTTCT TTATCATGCA
3001  ACTCGTAGGA CAGGTGCCGG CAGCGCTCTG GGTCATTTTC GGCGAGGACC
3051  GCTTTCGCTG GAGCGCGACG ATGATCGGCC TGTCGCTTGC GGTATTCGGA
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FIG. 20 (cont'd)

```
3101  ATCTTGCACG CCCTCGCTCA AGCCTTCGTC ACTGGTCCCG CCACCAAACG
3151  TTTCGGCGAG AAGCAGGCCA TTATCGCCGG CATGGCGGCC GACGCGCTGG
3201  GCTACGTCTT GCTGGCGTTC GCGACGCGAG GCTGGATGGC CTTCCCCATT
3251  ATGATTCTTC TCGCTTCCGG CGGCATCGGG ATGCCCGCGT TGCAGGCCAT
3301  GCTGTCCAGG CAGGTAGATG ACGACCATCA GGGACAGCTT CAAGGATCGC
3351  TCGCGGCTCT TACCAGCCTA ACTTCGATCA CTGGACCGCT GATCGTCACG
3401  GCGATTTATG CCGCCTCGGC GAGCACATGG AACGGGTTGG CATGGATTGT
3451  AGGCGCCGCC CTATACCTTG TCTGCCTCCC CGCGTTGCGT CGCGGTGCAT
3501  GCAGCCGGGC CACCTCGACC TGAATGGAAG CCGGCGGCAC CTCGCTAACG
3551  GATTCACCAC TCCAAGAATT GGAGCCAATC AATTCTTGCG GAGAACTGTG
3601  AATGCGCAAA CCAACCCTTG GCAGAACATA TCCATCGCGT CCGCCATCTC
3651  CAGCAGCCGC ACGCGGCGCA TCTCGGGCCG CGTTGCTGGC GTTTTTCCAT
3701  AGGCTCCGCC CCCCTGACGA GCATCACAAA AATCGACGCT CAAGTCAGAG
3751  GTGGCGAAAC CCGACAGGAC TATAAAGATA CCAGGCGTTT CCCCCTGGAA
3801  GCTCCCTCGT GCGCTCTCCT GTTCCGACCC TGCCGCTTAC CGGATACCTG
3851  TCCGCCTTTC TCCCTTCGGG AAGCGTGGCG CTTTCTCAAT GCTCACGCTG
3901  TAGGTATCTC AGTTCGGTGT AGGTCGTTCG CTCCAAGCTG GGCTGTGTGC
3951  ACGAACCCCC CGTTCAGCCC GACCGCTGCG CCTTATCCGG TAACTATCGT
4001  CTTGAGTCCA ACCCGGTAAG ACACGACTTA TCGCCACTGG CAGCAGCCAC
4051  TGGTAACAGG ATTAGCAGAG CGAGGTATGT AGGCGGTGCT ACAGAGTTCT
4101  TGAAGTGGTG GCCTAACTAC GGCTACACTA GAAGGACAGT ATTTGGTATC
4151  TGCGCTCTGC TGAAGCCAGT TACCTTCGGA AAAAGAGTTG GTAGCTCTTG
4201  ATCCGGCAAA CAAACCACCG CTGGTAGCGG TGGTTTTTTT GTTTGCAAGC
4251  AGCAGATTAC GCGCAGAAAA AAAGGATCTC AAGAAGATCC TTTGATCTTT
4301  TCTACGGGGT CTGACGCTCA GTGGAACGAA AACTCACGTT AAGGGATTTT
4351  GGTCATGAGA TTATCAAAAA GGATCTTCAC CTAGATCCTT TTAAATTAAA
4401  AATGAAGTTT TAAATCAATC TAAAGTATAT ATGAGTAAAC TTGGTCTGAC
4451  AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT
4501  TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC
4551  GGGAGGGCTT ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA
4601  CGCTCACCGG CTCCAGATTT ATCAGCAATA AACCAGCCAG CCGGAAGGGC
4651  CGAGCGCAGA AGTGGTCCTG CAACTTTATC CGCCTCCATC CAGTCTATTA
4701  ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA TAGTTTGCGC
```

FIG. 20 (cont'd)

```
4751  AACGTTGTTG CCATTGCTGC AGGCATCGTG GTGTCACGCT CGTCGTTTGG
4801  TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT
4851  CCCCCATGTT GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT
4901  GTCAGAAGTA AGTTGGCCGC AGTGTTATCA CTCATGGTTA TGGCAGCACT
4951  GCATAATTCT CTTACTGTCA TGCCATCCGT AAGATGCTTT TCTGTGACTG
5001  GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG GCGACCGAGT
5051  TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC
5101  TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA
5151  GGATCTTACC GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC
5201  AACTGATCTT CAGCATCTTT TACTTTCACC AGCGTTTCTG GGTGAGCAAA
5251  AACAGGAAGG CAAAATGCCG CAAAAAAGGG AATAAGGGCG ACACGGAAAT
5301  GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG CATTTATCAG
5351  GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA
5401  ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT
5451  AAGAAACCAT TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG
5501  AGGCCCTTTC GTCTTCAA
```

FIG. 21

```
pBG393
:BG368 backbone
:soluble T4#8
:AA #3 = LYS
:"perfect" Stu/first 182 AA of T4
:basically up to V2J2

      bg393.seq  Length: 5566

     1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
    51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
   101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
   151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
   201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
   251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
   301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
   351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
   401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
   451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
   501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
   551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
   601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
   651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
   701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
   751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
   801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
   851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
   901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
   951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
  1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
  1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
  1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
  1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
  1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
  1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
  1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
  1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
```

FIG. 21 (cont'd)

```
1401  GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
1451  CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501  CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551  GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601  ACCTGCTTCA GGGGCAGAGC CTGACCCTGA CCTTGGAGAG CCCCCCTGGT
1651  AGTAGCCCCT CAGTGCAATG TAGGAGTCCA AGGGGTAAAA ACATACAGGG
1701  GGGGAAGACC CTCTCCGTGT CTCAGCTGGA GCTCCAGGAT AGTGGCACCT
1751  GGACATGCAC TGTCTTGCAG AACCAGAAGA AGGTGGAGTT CAAAATAGAC
1801  ATCGTGGTGC TAGCTTTCCA GTGAGATCTT TGTGAAGGAA CCTTACTTCT
1851  GTGGTGTGAC ATAATTGGAC AAACTACCTA CAGAGATTTA AAGCTCTAAG
1901  GTAAATATAA AATTTTTAAG TGTATAATGT GTTAAACTAC TGATTCTAAT
1951  TGTTTGTGTA TTTTAGATTC CAACCTATGG AACTGATGAA TGGGAGCAGT
2001  GGTGGAATGC CTTTAATGAG GAAAACCTGT TTTGCTCAGA AGAAATGCCA
2051  TCTAGTGATG ATGAGGCTAC TGCTGACTCT CAACATTCTA CTCCTCCAAA
2101  AAAGAAGAGA AAGGTAGAAG ACCCCAAGGA CTTTCCTTCA GAATTGCTAA
2151  GTTTTTTGAG TCATGCTGTG TTTAGTAATA GAACTCTTGC TTGCTTTGCT
2201  ATTTACACCA CAAAGGAAAA AGCTGCACTG CTATACAAGA AAATTATGGA
2251  AAAATATTCT GTAACCTTTA TAAGTAGGCA TAACAGTTAT AATCATAACA
2301  TACTGTTTTT TCTTACTCCA CACAGGCATA GAGTGTCTGC TATTAATAAC
2351  TATGCTCAAA AATTGTGTAC CTTTAGCTTT TTAATTTGTA AAGGGGTTAA
2401  TAAGGAATAT TTGATGTATA GTGCCTTGAC TAGAGATCAT AATCAGCCAT
2451  ACCACATTTG TAGAGGTTTT ACTTGCTTTA AAAAACCTCC CACACCTCCC
2501  CCTGAACCTG AAACATAAAA TGAATGCAAT TGTTGTTGTT AACTTGTTTA
2551  TTGCAGCTTA TAATGGTTAC AAATAAAGCA ATAGCATCAC AAATTTCACA
2601  AATAAAGCAT TTTTTTCACT GCATTCTAGT TGTGGTTTGT CCAAACTCAT
2651  CAATGTATCT TATCATGTCT GGATCCTCTA CGCCGGACGC ATCGTGGCCG
2701  GCATCACCGG CGCCACAGGT GCGGTTGCTG GCGCCTATAT CGCCGACATC
2751  ACCGATGGGG AAGATCGGGC TCGCCACTTC GGGCTCATGA GCGCTTGTTT
2801  CGGCGTGGGT ATGGTGGCAG GCCCGTGGCC GGGGGACTGT TGGGCGCCAT
2851  CTCCTTGCAT GCACCATTCC TTGCGGCGGC GGTGCTCAAC GGCCTCAACC
2901  TACTACTGGG CTGCTTCCTA ATGCAGGAGT CGCATAAGGG AGAGCGTCGA
2951  CCGATGCCCT TGAGAGCCTT CAACCCAGTC AGCTCCTTCC GGTGGGCGCG
3001  GGGCATGACT ATCGTCGCCG CACTTATGAC TGTCTTCTTT ATCATGCAAC
```

FIG. 2I (cont'd)

```
3051  TCGTAGGACA GGTGCCGGCA GCGCTCTGGG TCATTTTCGG CGAGGACCGC
3101  TTTCGCTGGA GCGCGACGAT GATCGGCCTG TCGCTTGCGG TATTCGGAAT
3151  CTTGCACGCC CTCGCTCAAG CCTTCGTCAC TGGTCCCGCC ACCAAACGTT
3201  TCGGCGAGAA GCAGGCCATT ATCGCCGGCA TGGCGGCCGA CGCGCTGGGC
3251  TACGTCTTGC TGGCGTTCGC GACGCGAGGC TGGATGGCCT TCCCCATTAT
3301  GATTCTTCTC GCTTCCGGCG GCATCGGGAT GCCCGCGTTG CAGGCCATGC
3351  TGTCCAGGCA GGTAGATGAC GACCATCAGG GACAGCTTCA AGGATCGCTC
3401  GCGGCTCTTA CCAGCCTAAC TTCGATCACT GGACCGCTGA TCGTCACGGC
3451  GATTTATGCC GCCTCGGCGA GCACATGGAA CGGGTTGGCA TGGATTGTAG
3501  GCGCCGCCCT ATACCTTGTC TGCCTCCCCG CGTTGCGTCG CGGTGCATGG
3551  AGCCGGGCCA CCTCGACCTG AATGGAAGCC GGCGGCACCT CGCTAACGGA
3601  TTCACCACTC CAAGAATTGG AGCCAATCAA TTCTTGCGGA GAACTGTGAA
3651  TGCGCAAACC AACCCTTGGC AGAACATATC CATCGCGTCC GCCATCTCCA
3701  GCAGCCGCAC GCGGCGCATC TCGGGCCGCG TTGCTGGCGT TTTTCCATAG
3751  GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT
3801  GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC
3851  TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC
3901  CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCAATGC TCACGCTGTA
3951  GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC
4001  GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT
4051  TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG
4101  GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG
4151  AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG
4201  CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT
4251  CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG
4301  CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC
4351  TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG
4401  TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA
4451  TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG
4501  TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC
4551  GTTCATCCAT AGTTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG
4601  GAGGGCTTAC CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG
4651  CTCACCGGCT CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG
```

FIG. 21 (cont'd)

```
4701  AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT
4751  TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA
4801  CGTTGTTGCC ATTGCTGCAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA
4851  TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC
4901  CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT
4951  CAGAAGTAAG TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC
5001  ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT
5051  GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG
5101  CTCTTGCCCG GCGTCAACAC GGGATAATAC CGCGCCACAT AGCAGAACTT
5151  TAAAAGTGCT CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG
5201  ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA
5251  CTGATCTTCA GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA
5301  CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT
5351  TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG
5401  TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC
5451  AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA
5501  GAAACCATTA TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG
5551  GCCCTTTCGT CTTCAA
```

FIG. 22

```
pBG395
:BG368 backbone
:soluble T4#10
:AA #3 = LYS
:first 131 AA of T4

      pg395.seq  Length: 5413

   1  GAATTAATTC CAGCTTGCTG TGGAATGTGT GTCAGTTAGG GTGTGGAAAG
  51  TCCCCAGGCT CCCCAGCAGG CAGAAGTATG CAAAGCATGC ATCTCAATTA
 101  GTCAGCAACC AGGTGTGGAA AGTCCCCAGG CTCCCCAGCA GGCAGAAGTA
 151  TGCAAAGCAT GCATCTCAAT TAGTCAGCAA CCATAGTCCC GCCCCTAACT
 201  CCGCCCATCC CGCCCCTAAC TCCGCCCAGT TCCGCCCATT CTCCGCCCCA
 251  TGGCTGACTA ATTTTTTTTA TTTATGCAGA GGCCGAGGCC GCCTCGGCCT
 301  CTGAGCTATT CCAGAAGTAG TGAGGAGGCT TTTTTGGAGG GGTCCTCCTC
 351  GTATAGAAAC TCGGACCACT CTGAGACGAA GGCTCGCGTC CAGGCCAGCA
 401  CGAAGGAGGC TAAGTGGGAG GGGTAGCGGT CGTTGTCCAC TAGGGGGTCC
 451  ACTCGCTCCA GGGTGTGAAG ACACATGTCG CCCTCTTCGG CATCAAGGAA
 501  GGTGATTGGT TTATAGGTGT AGGCCACGTG ACCGGGTGTT CCTGAAGGGG
 551  GGCTATAAAA GGGGGTGGGG GCGCGTTCGT CCTCACTCTC TTCCGCATCG
 601  CTGTCTGCGA GGGCCAGCTG TTGGGCTCGC GGTTGAGGAC AAACTCTTCG
 651  CGGTCTTTCC AGTACTCTTG GATCGGAAAC CCGTCGGCCT CCGAACGGTA
 701  CTCCGCCACC GAGGGACCTG AGCGAGTCCG CATCGACCGG ATCGGAAAAC
 751  CTCTCGAGAA AGGCGTCTAA CCAGTCACAG TCGCAAGGTA GGCTGAGCAC
 801  CGTGGCGGGC GGCAGCGGGT GGCGGTCGGG GTTGTTTCTG GCGGAGGTGC
 851  TGCTGATGAT GTAATTAAAG TAGGCGGTCT TGAGACGGCG GATGGTCGAG
 901  GTGAGGTGTG GCAGGCTTGA GATCGATCTG GCCATACACT TGAGTGACAA
 951  TGACATCCAC TTTGCCTTTC TCTCCACAGG TGTCCACTCC CAGGTCCAAC
1001  TGGATCCAAG CTTCGACTCG AGGAATTCCC CGAAGGAACA AAGCACCCTC
1051  CCCACTGGGC TCCTGGTTGC AGAGCTCCAA GTCCTCACAC AGATACGCCT
1101  GTTTGAGAAG CAGCGGGCAA GAAAGACGCA AGCCCAGAGG CCCTGCCATT
1151  TCTGTGGGCT CAGGTCCCTA CTGGCTCAGG CCCCTGCCTC CCTCGGCAAG
1201  GCCACAATGA ACCGGGGAGT CCCTTTTAGG CACTTGCTTC TGGTGCTGCA
1251  ACTGGCGCTC CTCCCAGCAG CCACTCAGGG AAAGAAAGTG GTGCTGGGCA
1301  AAAAAGGGGA TACAGTGGAA CTGACCTGTA CAGCTTCCCA GAAGAAGAGC
1351  ATACAATTCC ACTGGAAAAA CTCCAACCAG ATAAAGATTC TGGGAAATCA
1401  GGGCTCCTTC TTAACTAAAG GTCCATCCAA GCTGAATGAT CGCGCTGACT
```

FIG. 22(cont'd)

```
1451  CAAGAAGAAG CTTGTGGGAC CAAGGAAACT TTCCCCTGAT CATCAAGAAT
1501  CTTAAGATAG AAGACTCAGA TACTTACATC TGTGAAGTGG AGGACCAGAA
1551  GGAGGAGGTG CAATTGCTAG TGTTCGGATT GACTGCCAAC TCTGACACCC
1601  ACCTGCTTCA GGGGCAGAGC CTGACCCTGA CCTTGGAGAG CCCCCCGGGT
1651  AGTAGCCCCT CAGTGCAATG AGATCTTTGT GAAGGAACCT TACTTCTGTG
1701  GTGTGACATA ATTGGACAAA CTACCTACAG AGATTTAAAG CTCTAAGGTA
1751  AATATAAAAT TTTTAAGTGT ATAATGTGTT AAACTACTGA TTCTAATTGT
1801  TTGTGTATTT TAGATTCCAA CCTATGGAAC TGATGAATGG GAGCAGTGGT
1851  GGAATGCCTT TAATGAGGAA AACCTGTTTT GCTCAGAAGA AATGCCATCT
1901  AGTGATGATG AGGCTACTGC TGACTCTCAA CATTCTACTC CTCCAAAAAA
1951  GAAGAGAAAG GTAGAAGACC CCAAGGACTT TCCTTCAGAA TTGCTAAGTT
2001  TTTTGAGTCA TGCTGTGTTT AGTAATAGAA CTCTTGCTTG CTTTGCTATT
2051  TACACCACAA AGGAAAAAGC TGCACTGCTA TACAAGAAAA TTATGGAAAA
2101  ATATTCTGTA ACCTTTATAA GTAGGCATAA CAGTTATAAT CATAACATAC
2151  TGTTTTTTCT TACTCCACAC AGGCATAGAG TGTCTGCTAT TAATAACTAT
2201  GCTCAAAAAT TGTGTACCTT TAGCTTTTTA ATTTGTAAAG GGGTTAATAA
2251  GGAATATTTG ATGTATAGTG CCTTGACTAG AGATCATAAT CAGCCATACC
2301  ACATTTGTAG AGGTTTTACT TGCTTTAAAA AACCTCCCAC ACCTCCCCCT
2351  GAACCTGAAA CATAAAATGA ATGCAATTGT TGTTGTTAAC TTGTTTATTG
2401  CAGCTTATAA TGGTTACAAA TAAAGCAATA GCATCACAAA TTTCACAAAT
2451  AAAGCATTTT TTTCACTGCA TTCTAGTTGT GGTTTGTCCA AACTCATCAA
2501  TGTATCTTAT CATGTCTGGA TCCTCTACGC CGGACGCATC GTGGCCGGCA
2551  TCACCGGCGC CACAGGTGCG GTTGCTGGCG CCTATATCGC CGACATCACC
2601  GATGGGGAAG ATCGGGCTCG CCACTTCGGG CTCATGAGCG CTTGTTTCGG
2651  CGTGGGTATG GTGGCAGGCC CGTGGCCGGG GGACTGTTGG GCGCCATCTC
2701  CTTGCATGCA CCATTCCTTG CGGCGGCGGT GCTCAACGGC CTCAACCTAC
2751  TACTGGGCTG CTTCCTAATG CAGGAGTCGC ATAAGGGAGA GCGTCGACCG
2801  ATGCCCTTGA GAGCCTTCAA CCCAGTCAGC TCCTTCCGGT GGGCGCGGGG
2851  CATGACTATC GTCGCCGCAC TTATGACTGT CTTCTTTATC ATGCAACTCG
2901  TAGGACAGGT GCCGGCAGCG CTCTGGGTCA TTTTCGGCGA GGACCGCTTT
2951  CGCTGGAGCG CGACGATGAT CGGCCTGTCG CTTGCGGTAT TCGGAATCTT
3001  GCACGCCCTC GCTCAAGCCT TCGTCACTGG TCCCGCCACC AAACGTTTCG
3051  GCGAGAAGCA GGCCATTATC GCCGGCATGG CGGCCGACGC GCTGGGCTAC
```

FIG. 22(cont'd)

```
3101  GTCTTGCTGG CGTTCGCGAC GCGAGGCTGG ATGGCCTTCC CCATTATGAT
3151  TCTTCTCGCT TCCGGCGGCA TCGGGATGCC CGCGTTGCAG GCCATGCTGT
3201  CCAGGCAGGT AGATGACGAC CATCAGGGAC AGCTTCAAGG ATCGCTCGCG
3251  GCTCTTACCA GCCTAACTTC GATCACTGGA CCGCTGATCG TCACGGCGAT
3301  TTATGCCGCC TCGGCGAGCA CATGGAACGG GTTGGCATGG ATTGTAGGCG
3351  CCGCCCTATA CCTTGTCTGC CTCCCCGCGT TGCGTCGCGG TGCATGGAGC
3401  CGGGCCACCT CGACCTGAAT GGAAGCCGGC GGCACCTCGC TAACGGATTC
3451  ACCACTCCAA GAATTGGAGC CAATCAATTC TTGCGGAGAA CTGTGAATGC
3501  GCAAACCAAC CCTTGGCAGA ACATATCCAT CGCGTCCGCC ATCTCCAGCA
3551  GCCGCACGCG GCGCATCTCG GGCCGCGTTG CTGGCGTTTT TCCATAGGCT
3601  CCGCCCCCCT GACGAGCATC ACAAAAATCG ACGCTCAAGT CAGAGGTGGC
3651  GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC TGGAAGCTCC
3701  CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT ACCTGTCCGC
3751  CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCAATGCTCA CGCTGTAGGT
3801  ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG TGTGCACGAA
3851  CCCCCCGTTC AGCCCGACCG CTGCGCCTTA TCCGGTAACT ATCGTCTTGA
3901  GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA GCCACTGGTA
3951  ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA GTTCTTGAAG
4001  TGGTGGCCTA ACTACGGCTA CACTAGAAGG ACAGTATTTG GTATCTGCGC
4051  TCTGCTGAAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC TCTTGATCCG
4101  GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTTGTTTG CAAGCAGCAG
4151  ATTACGCGCA GAAAAAAAGG ATCTCAAGAA GATCCTTTGA TCTTTTCTAC
4201  GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAAGGG ATTTTGGTCA
4251  TGAGATTATC AAAAAGGATC TTCACCTAGA TCCTTTTAAA TTAAAAATGA
4301  AGTTTTAAAT CAATCTAAAG TATATATGAG TAAACTTGGT CTGACAGTTA
4351  CCAATGCTTA ATCAGTGAGG CACCTATCTC AGCGATCTGT CTATTTCGTT
4401  CATCCATAGT TGCCTGACTC CCCGTCGTGT AGATAACTAC GATACGGGAG
4451  GGCTTACCAT CTGGCCCCAG TGCTGCAATG ATACCGCGAG ACCCACGCTC
4501  ACCGGCTCCA GATTTATCAG CAATAAACCA GCCAGCCGGA AGGGCCGAGC
4551  GCAGAAGTGG TCCTGCAACT TTATCCGCCT CCATCCAGTC TATTAATTGT
4601  TGCCGGGAAG CTAGAGTAAG TAGTTCGCCA GTTAATAGTT TGCGCAACGT
4651  TGTTGCCATT GCTGCAGGCA TCGTGGTGTC ACGCTCGTCG TTTGGTATGG
4701  CTTCATTCAG CTCCGGTTCC CAACGATCAA GGCGAGTTAC ATGATCCCCC
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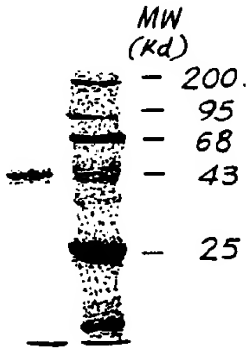
FIG. 22(cont'd)

```
4751  ATGTTGTGCA AAAAAGCGGT TAGCTCCTTC GGTCCTCCGA TCGTTGTCAG
4801  AAGTAAGTTG GCCGCAGTGT TATCACTCAT GGTTATGGCA GCACTGCATA
4851  ATTCTCTTAC TGTCATGCCA TCCGTAAGAT GCTTTTCTGT GACTGGTGAG
4901  TACTCAACCA AGTCATTCTG AGAATAGTGT ATGCGGCGAC CGAGTTGCTC
4951  TTGCCCGGCG TCAACACGGG ATAATACCGC GCCACATAGC AGAACTTTAA
5001  AAGTGCTCAT CATTGGAAAA CGTTCTTCGG GGCGAAAACT CTCAAGGATC
5051  TTACCGCTGT TGAGATCCAG TTCGATGTAA CCCACTCGTG CACCCAACTG
5101  ATCTTCAGCA TCTTTTACTT TCACCAGCGT TTCTGGGTGA GCAAAAACAG
5151  GAAGGCAAAA TGCCGCAAAA AAGGGAATAA GGGCGACACG GAAATGTTGA
5201  ATACTCATAC TCTTCCTTTT TCAATATTAT TGAAGCATTT ATCAGGGTTA
5251  TTGTCTCATG AGCGGATACA TATTTGAATG TATTTAGAAA AATAAACAAA
5301  TAGGGGTTCC GCGCACATTT CCCCGAAAAG TGCCACCTGA CGTCTAAGAA
5351  ACCATTATTA TCATGACATT AACCTATAAA AATAGGCGTA TCACGAGGCC
5401  CTTTCGTCTT CAA
```

FIG. 23



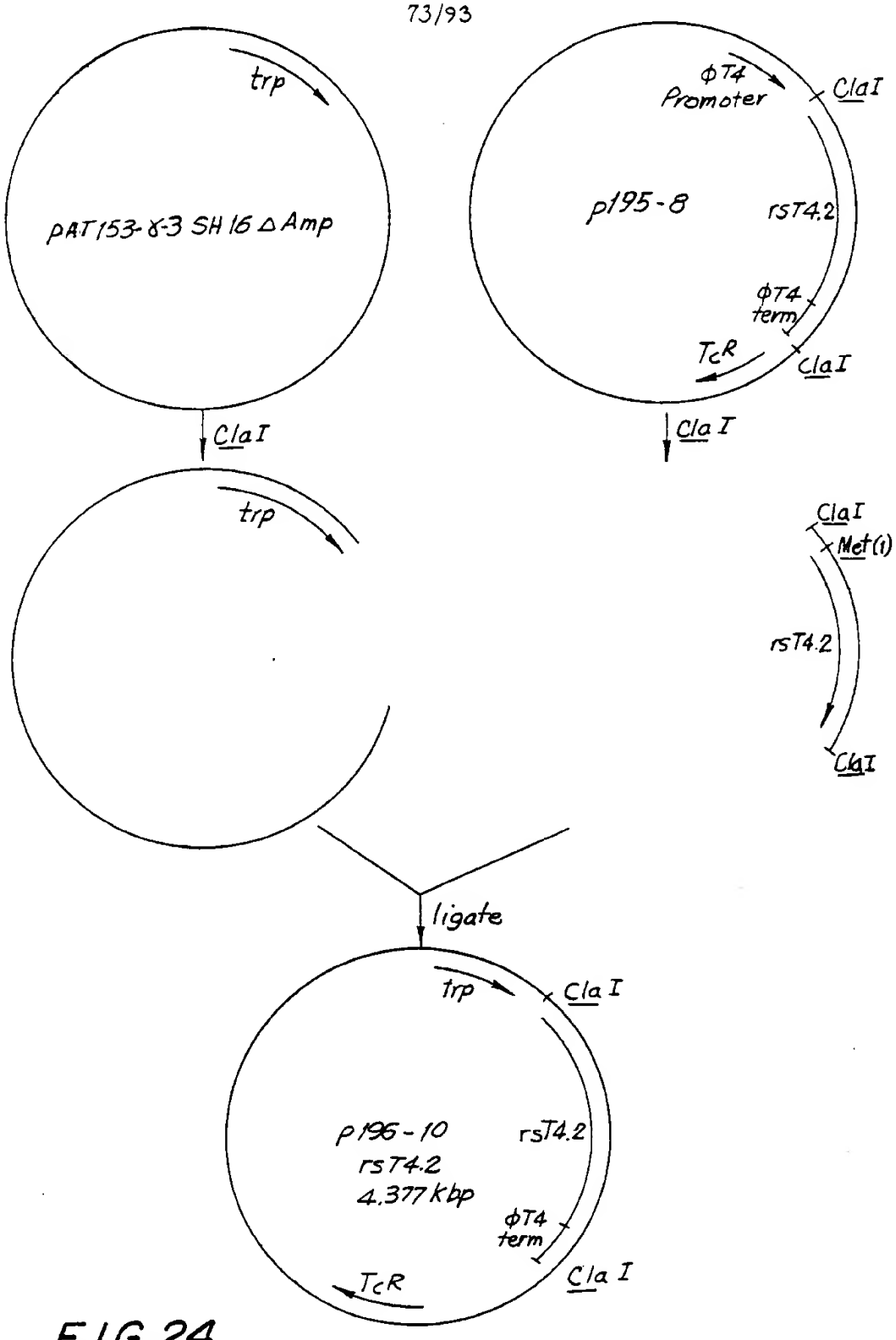

trp
pAT153-8-3 SH 16 Δ Amp
ClaI
ϕT4
Promoter
ClaI
p195-8
rsT4.2
ϕT4
term
TcR
ClaI
ClaI
trp
ClaI
Met(1)
rsT4.2
ClaI
ligate
trp
ClaI
p196-10
rsT4.2
4.377 kbp
rsT4.2
ϕT4
term
TcR
ClaI

FIG. 24

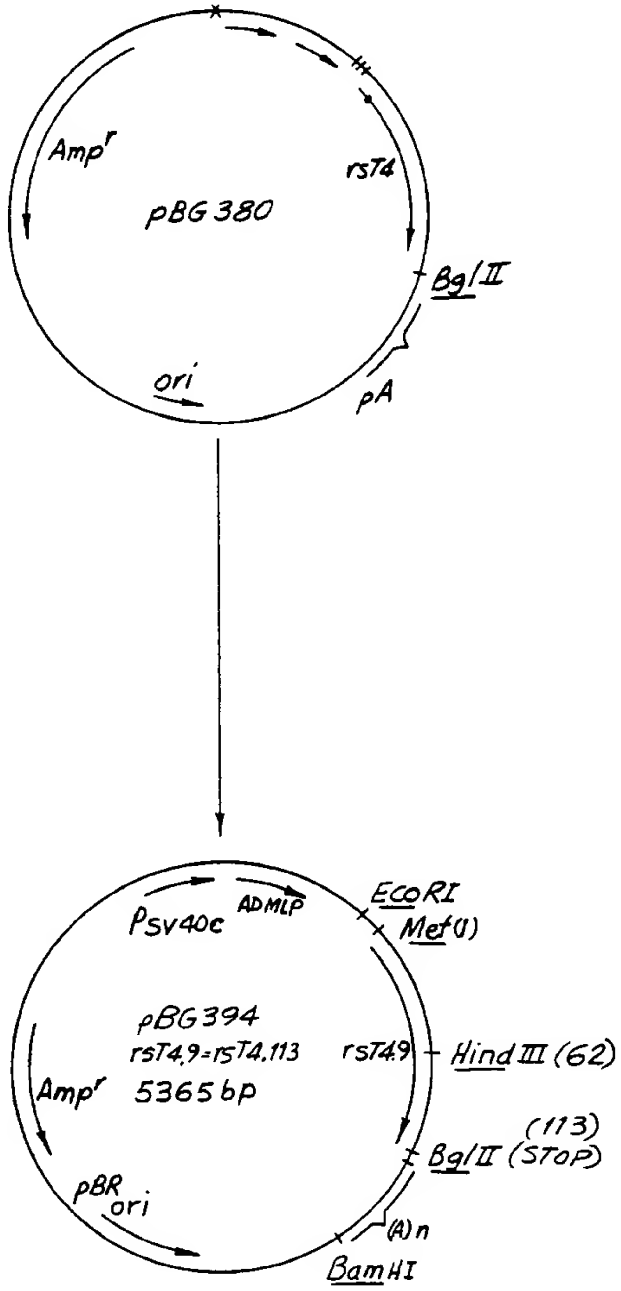

Amp^r
rsT4
pBG380
Bg/II
ori
pA
P SV40c
ADMLP
EcoRI
Met(1)
pBG394
rsT4.9-rsT4.113
rsT4.9
Hind III (62)
Amp^r
5365 bp
(113)
Bg/II (STOP)
pBR ori
(A)n
BamHI

FIG. 25

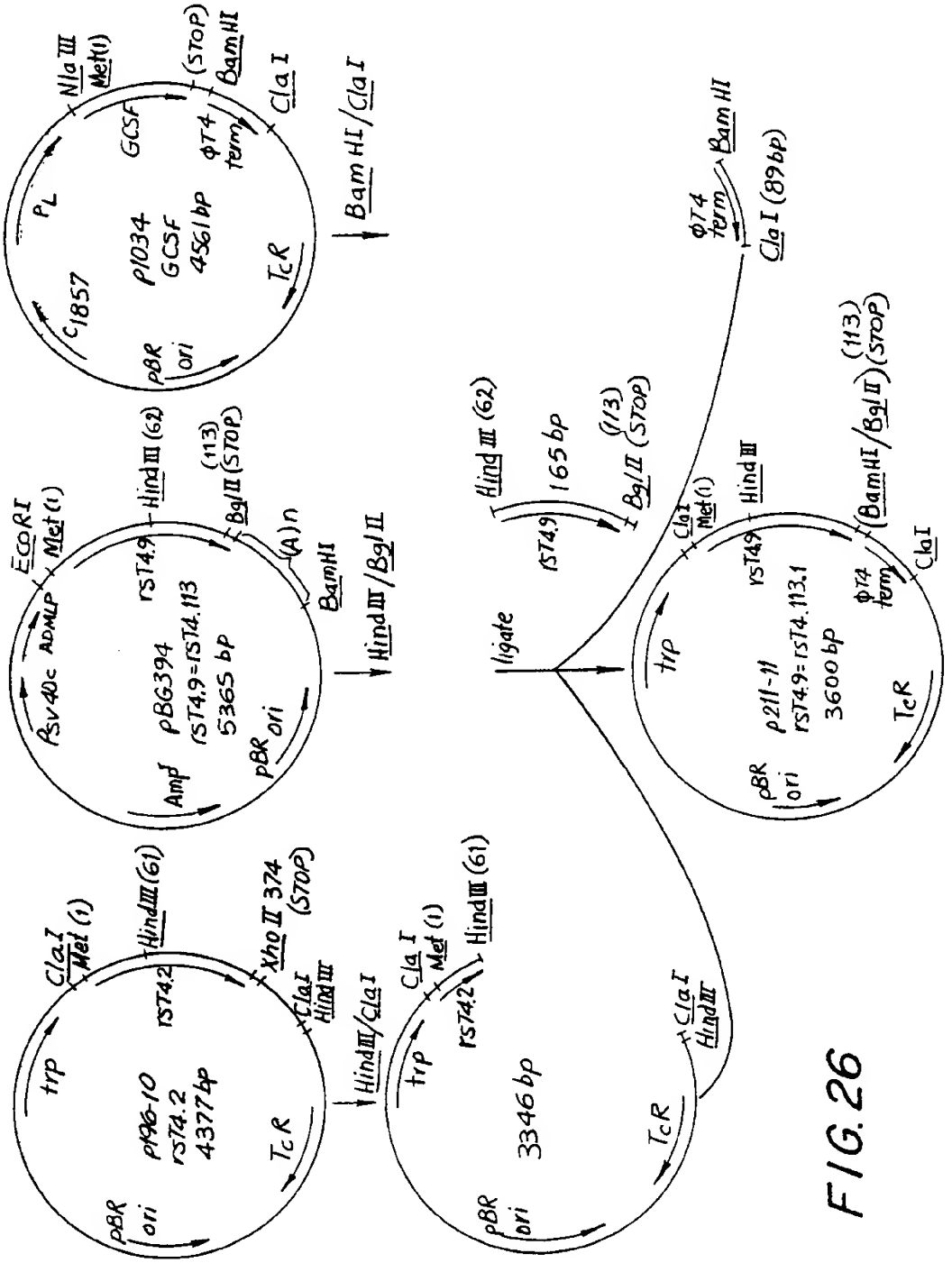

pBR ori
pl96-10
rST4.2
4377 bp
trp
TcR
ClaI Met (1)
HindIII (61)
XhoII 374 (STOP)
ClaI HindIII
HindIII/ClaI
pBR ori
3346 bp
trp
TcR
ClaI Met (1)
rST42
HindIII (61)
ClaI HindIII
pBR ori
pBG394
rST4.9=rST4.113
5365 bp
Amp
PSV40c ADMLP
EcoRI Met (1)
rST4.9
HindIII (62)
BglII (113) (STOP)
(A)n
BamHI
HindIII/BglII
pBR ori
p1034
GCSF
4561 bp
cI857
PL
NlaIII Met (1)
GCSF
(STOP) BamHI
φT4 term
ClaI
TcR
BamHI/ClaI
ligate
HindIII (62)
rST4.9
165 bp
BglII (113) (STOP)
pBR ori
p211-11
rST4.9=rST4.113.1
3600 bp
trp
TcR
ClaI Met (1)
rST4.9
HindIII
(BamHI/BglII) (113) (STOP)
φT4 term
ClaI
φT4 term
BamHI
ClaI (89 bp)

FIG. 26

FIG. 27

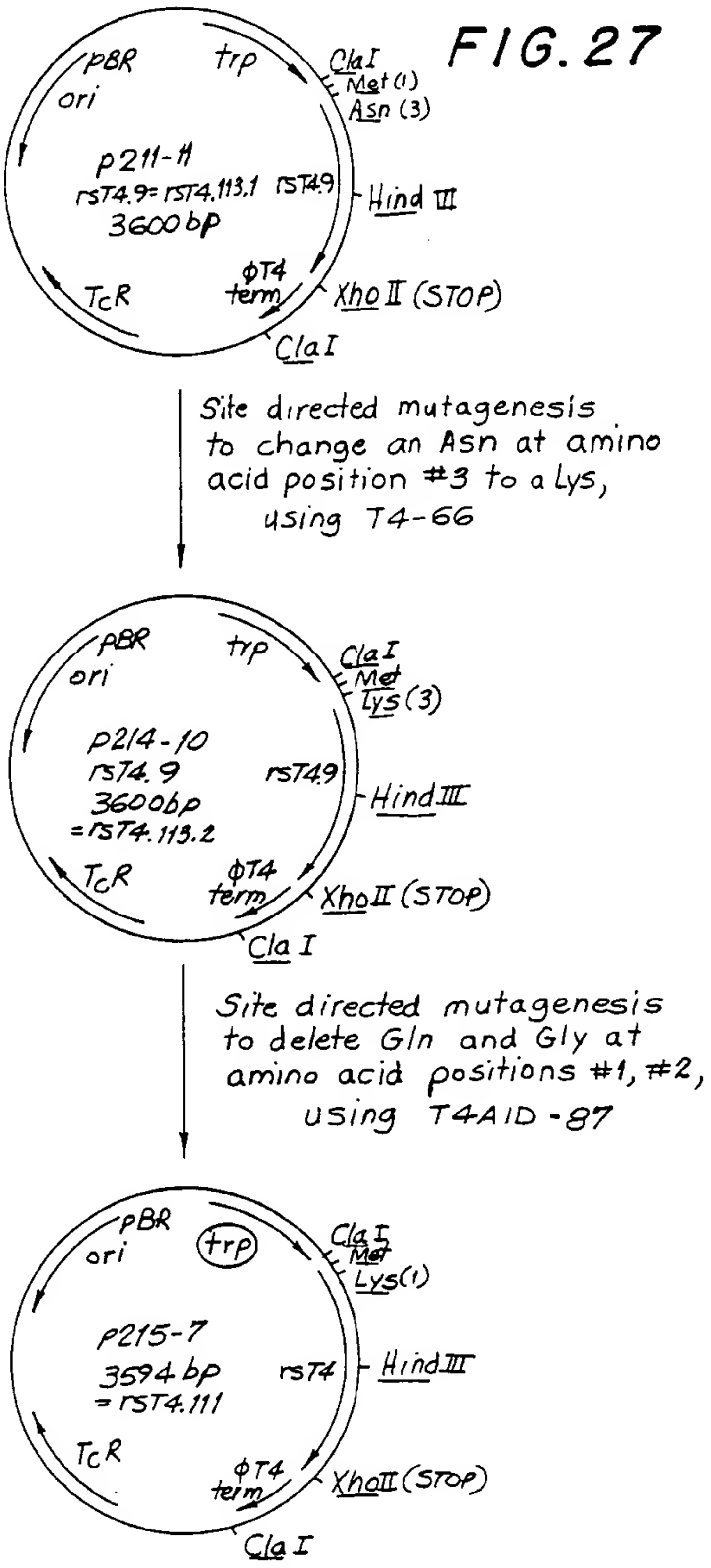
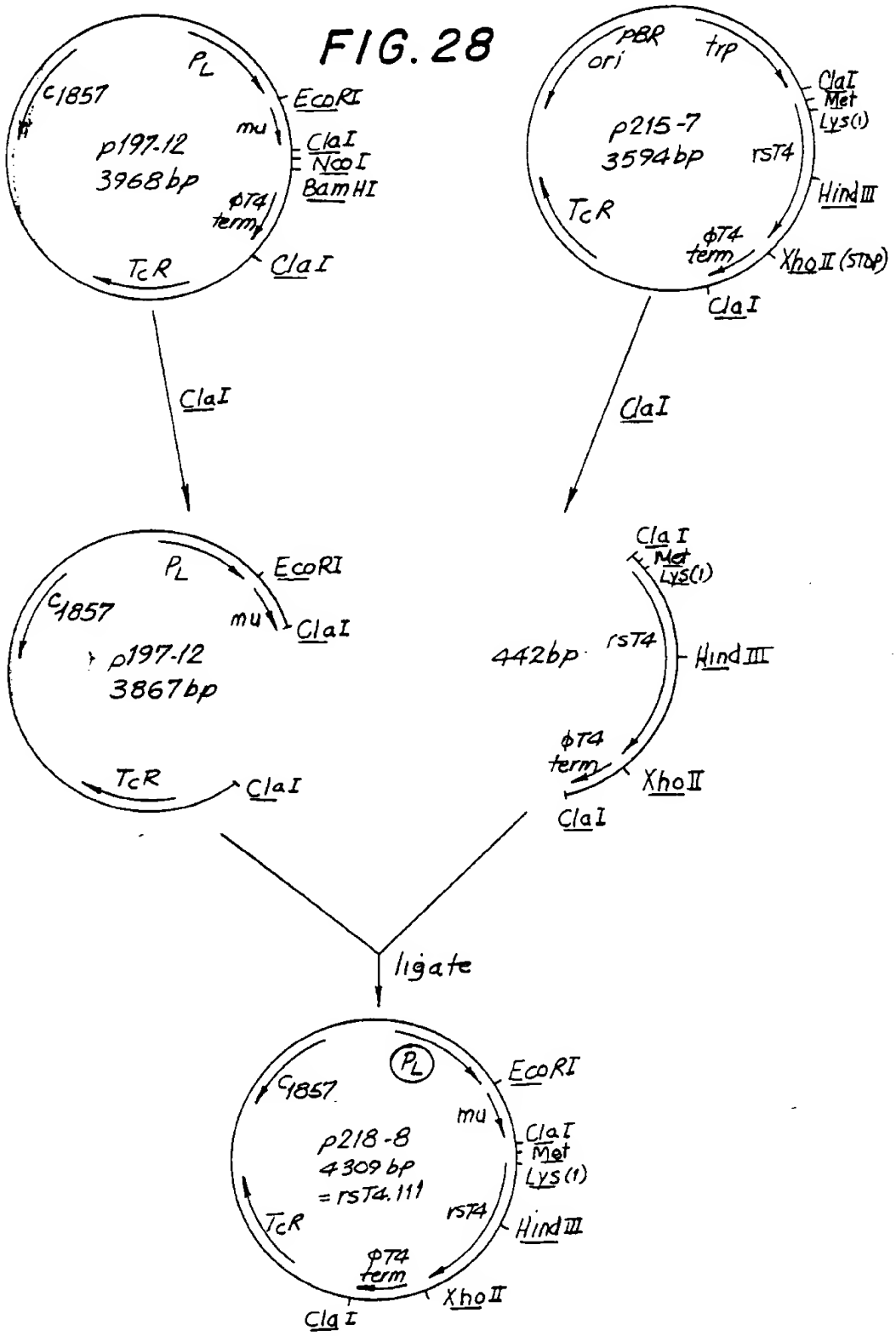

pBR ori
trp
ClaI
Met (1)
Asn (3)
p211-11
rsT4.9 = rsT4.113.1
rsT4.9
3600 bp
HindIII
TcR
ϕT4 term
XhoII (STOP)
ClaI
Site directed mutagenesis to change an Asn at amino acid position #3 to a Lys, using T4-66
pBR ori
trp
ClaI
Met
Lys (3)
p214-10
rsT4.9
3600bp
= rsT4.113.2
rsT4.9
HindIII
TcR
ϕT4 term
XhoII (STOP)
ClaI
Site directed mutagenesis to delete Gln and Gly at amino acid positions #1, #2, using T4AID-87
pBR ori
trp
ClaI
Met
Lys (1)
p215-7
3594 bp
= rsT4.111
rsT4
HindIII
TcR
ϕT4 term
XhoII (STOP)
ClaI

FIG. 28


P_L
c1857
EcoRI
mu
ClaI
NcoI
BamHI
p197.12
3968bp
φT4 term
TcR
ClaI
pBR ori
trp
ClaI
Met
Lys(1)
p215-7
3594bp
rsT4
HindIII
TcR
φT4 term
XhoII (STOP)
ClaI
ClaI
ClaI
P_L
EcoRI
c1857
mu
ClaI
p197-12
3867bp
TcR
ClaI
ClaI
Met
Lys(1)
442bp
rsT4
HindIII
φT4 term
XhoII
ClaI
ligate
P_L
EcoRI
c1857
mu
ClaI
Met
Lys(1)
p218-8
4309 bp
= rsT4.111
TcR
rsT4
HindIII
φT4 term
XhoII
ClaI

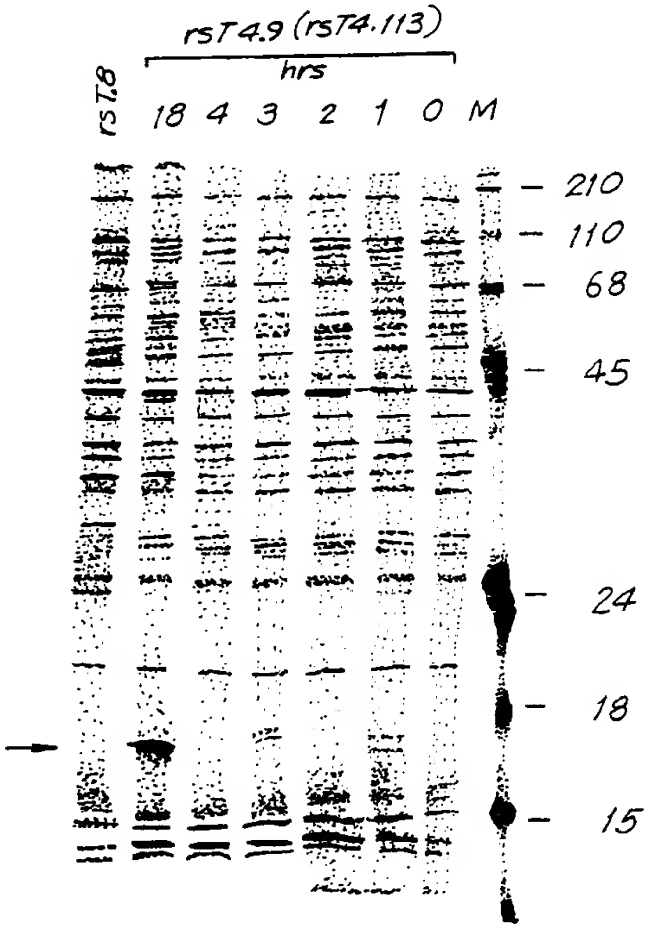


FIG. 29A

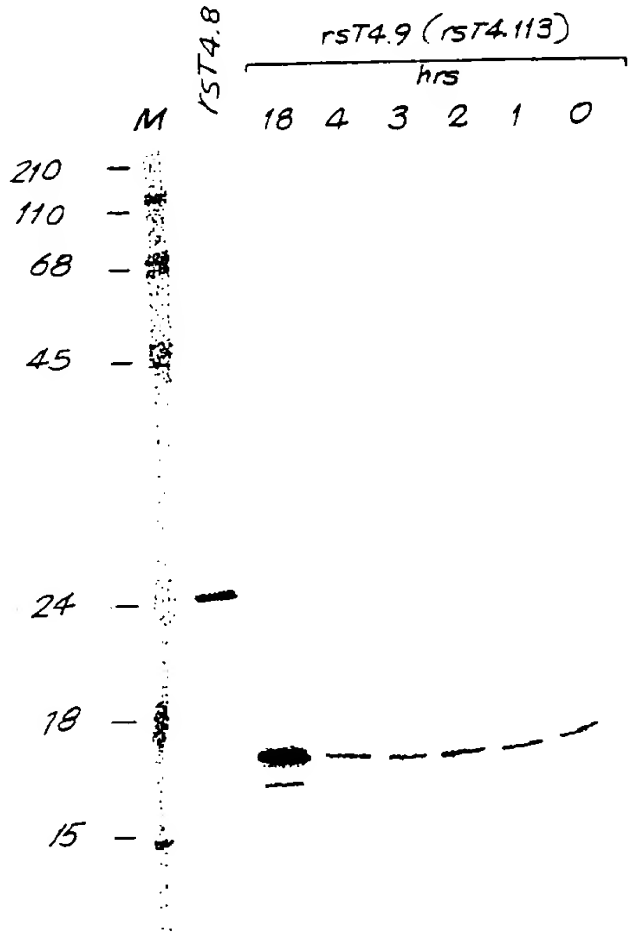
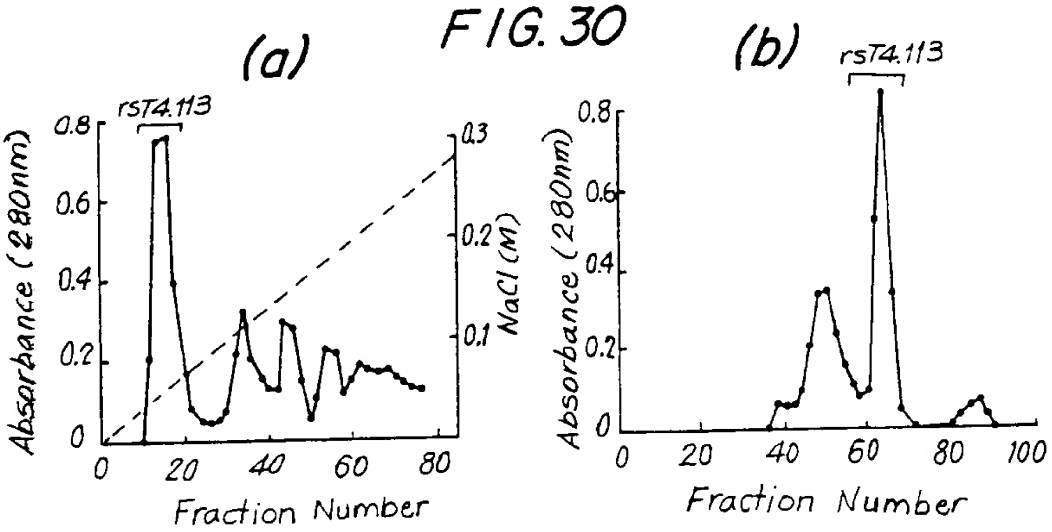
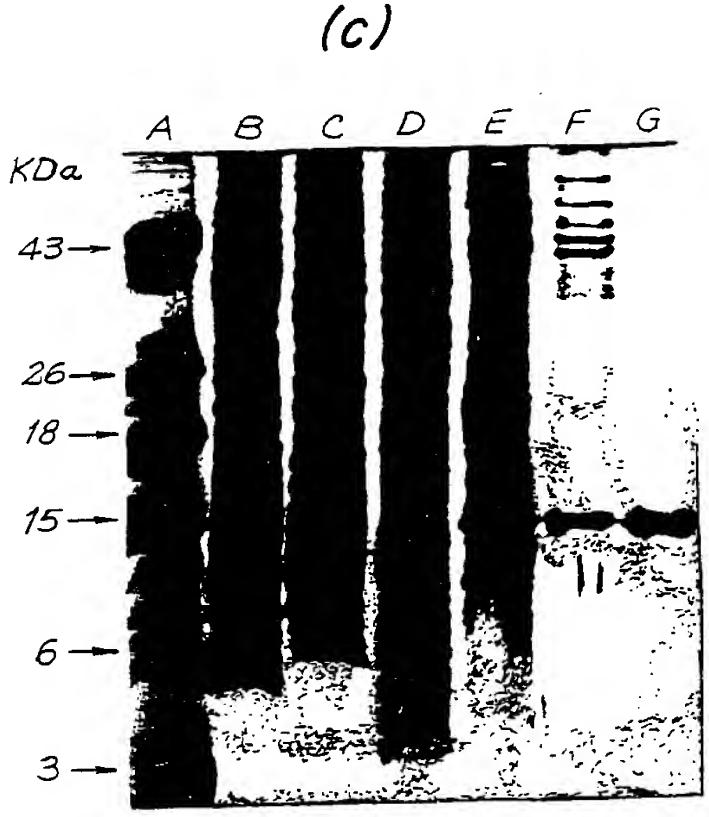


FIG. 29B

FIG. 30


(a)
rsT4.113
Absorbance (280nm)
0.8
0.6
0.4
0.2
0
0.3
0.2
0.1
NaCl (M)
0
20
40
60
80
Fraction Number
(b)
rsT4.113
Absorbance (280nm)
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
Fraction Number


(c)
A
B
C
D
E
F
G
KDa
43
26
18
15
6
3

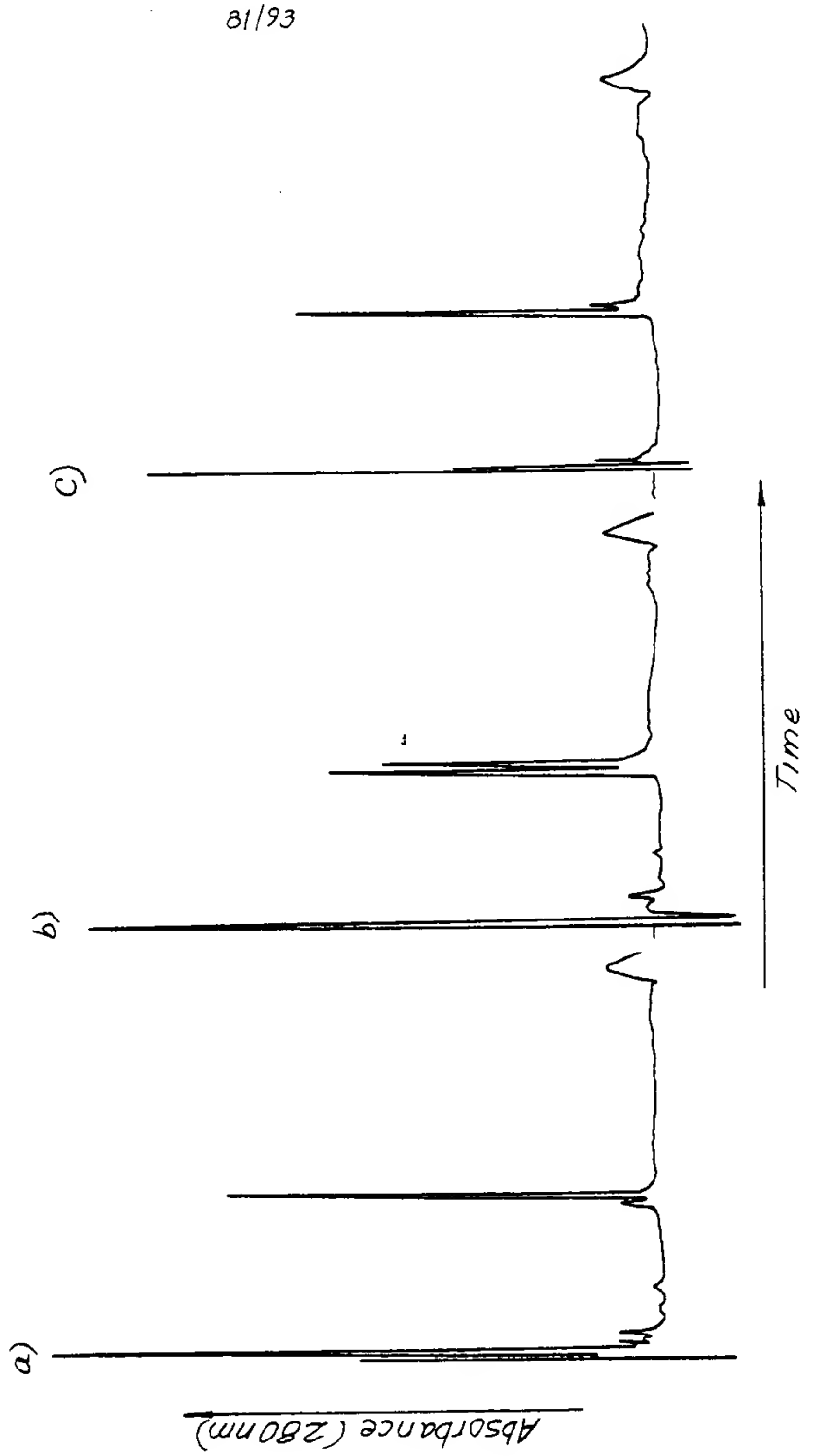


FIG. 31

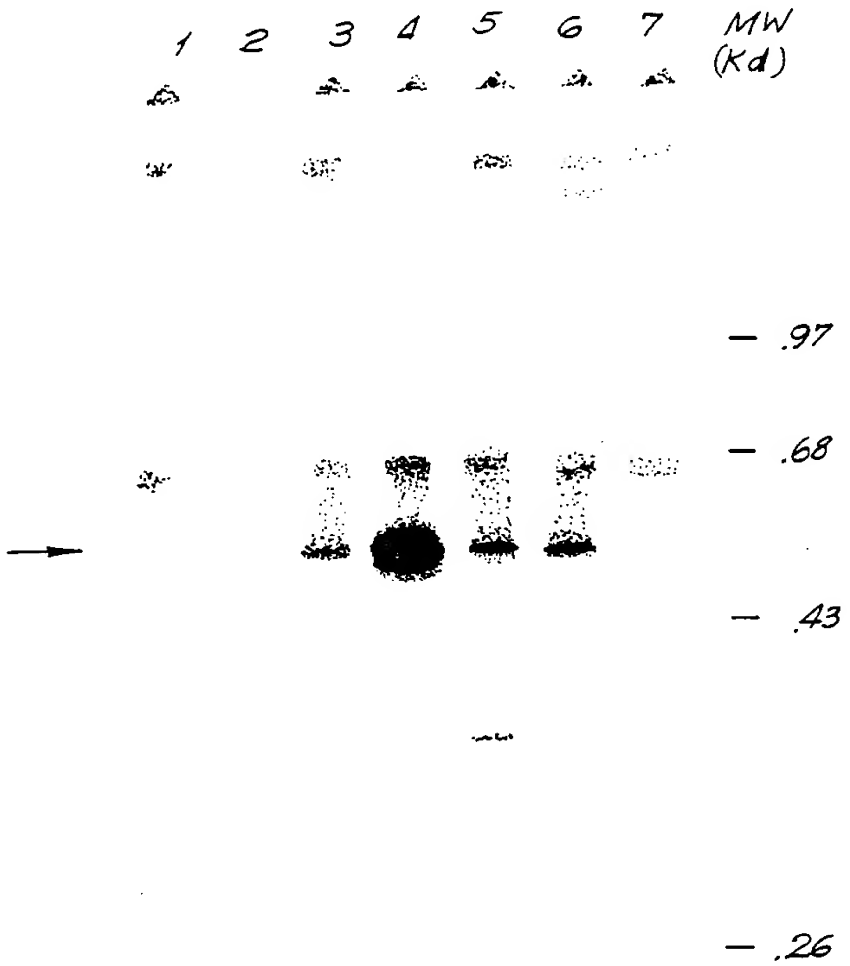


FIG. 32

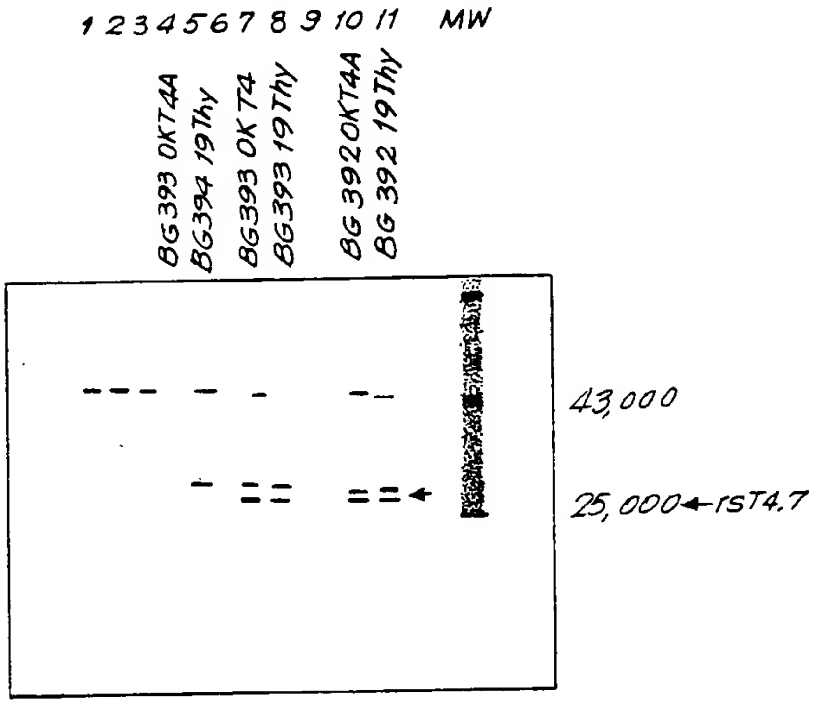

1 2 3 4 5 6 7 8 9 10 11 MW
BG 393 OKT4A
BG394 19Thy
BG393 OK T4
BG393 19Thy
BG 3920KT4A
BG 392 19Thy
43,000
25,000←1ST4.7

FIG.33

FIG. 34

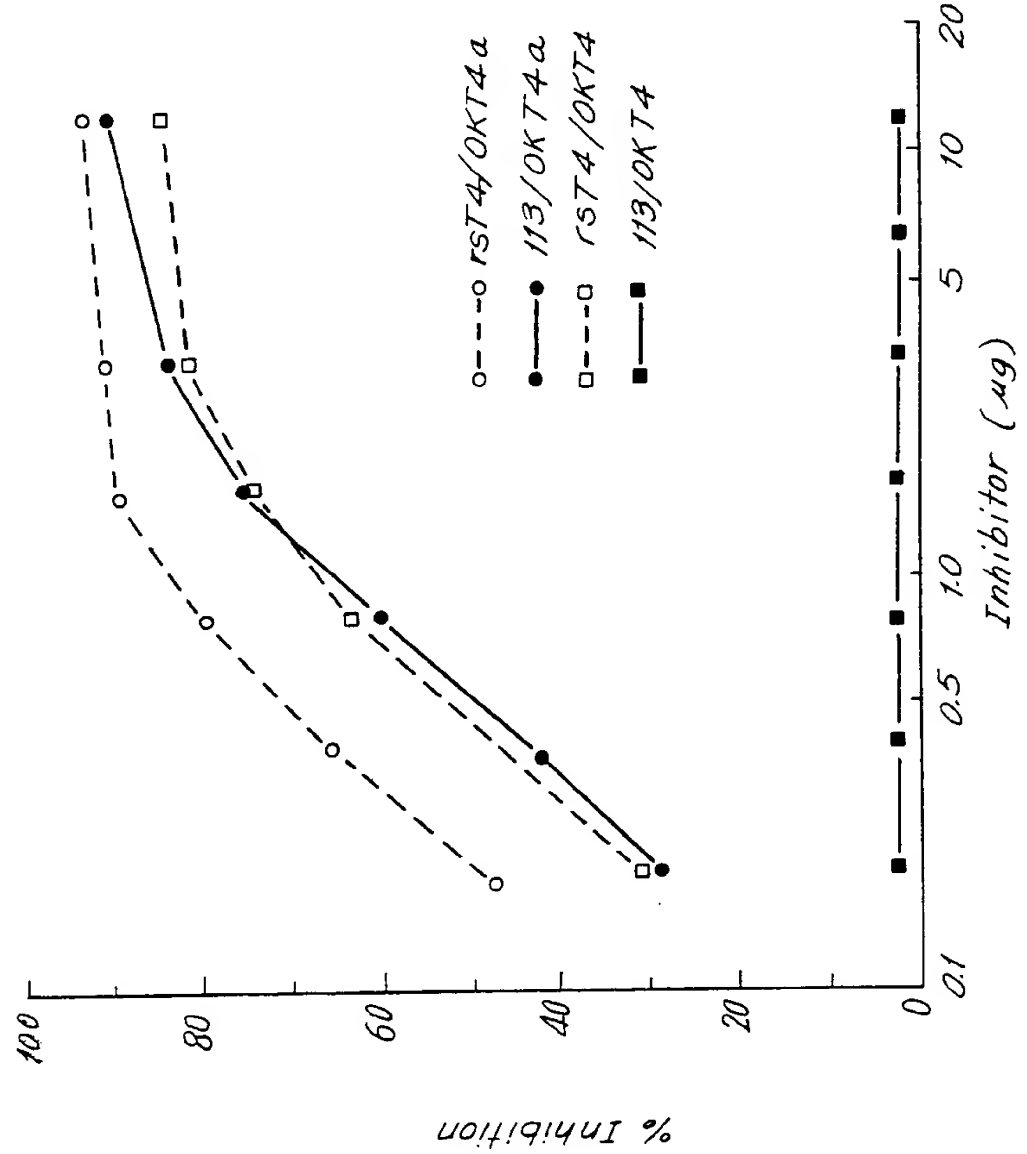

rsT4/OKT4a
113/OKT4a
rsT4/OKT4
113/OKT4
% Inhibition
100
80
60
40
20
0
.01
0.5
1.0
5
10
20
Inhibitor (μg)

FIG. 35

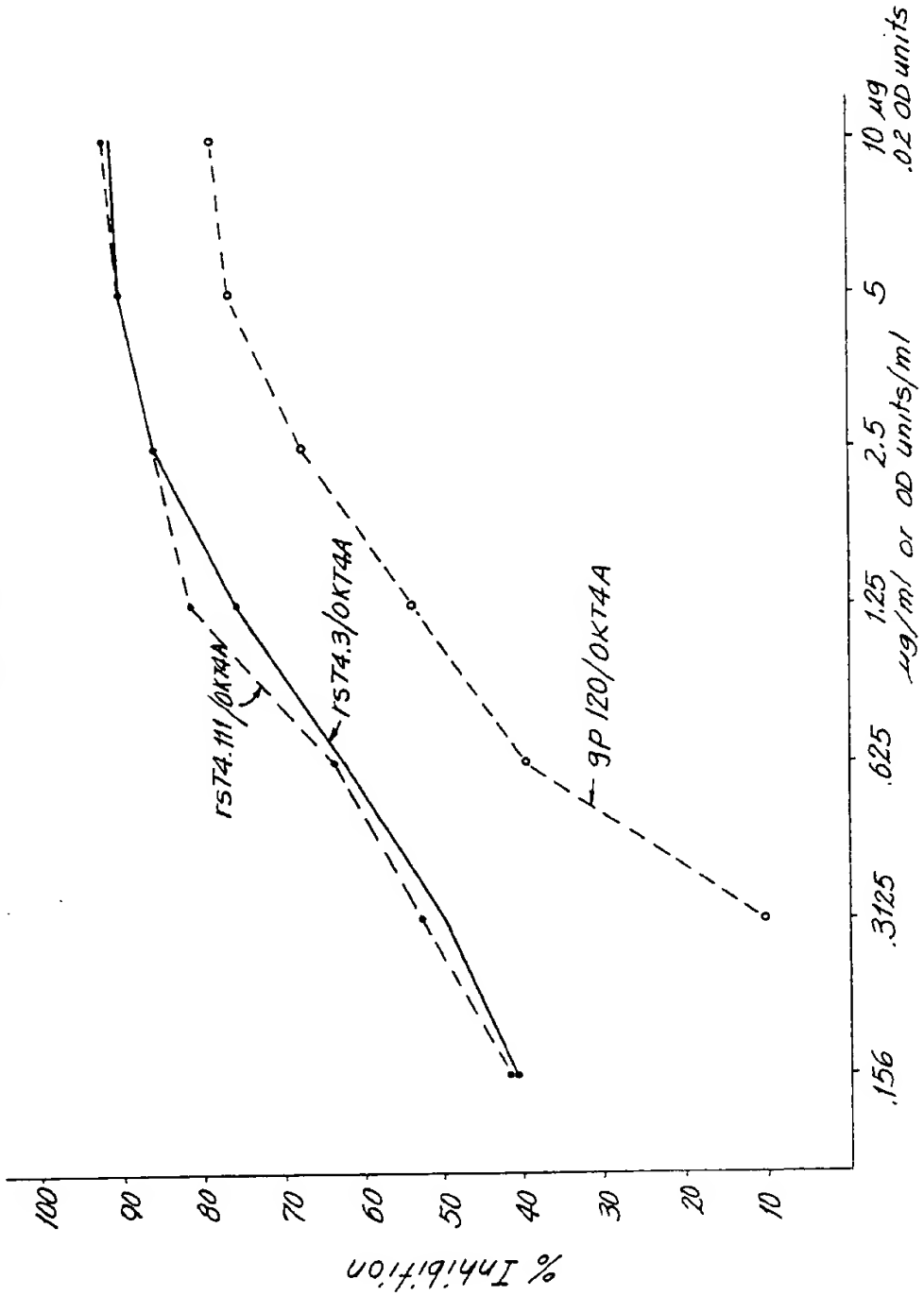

% Inhibition
100
90
80
70
60
50
40
30
20
10
.156
.3125
.625
1.25
2.5
5
10 µg
.02 OD units
µg/ml or OD units/ml
rs74.111/OKT4A
rs74.3/OKT4A
9P120/OKT4A

FIG. 36

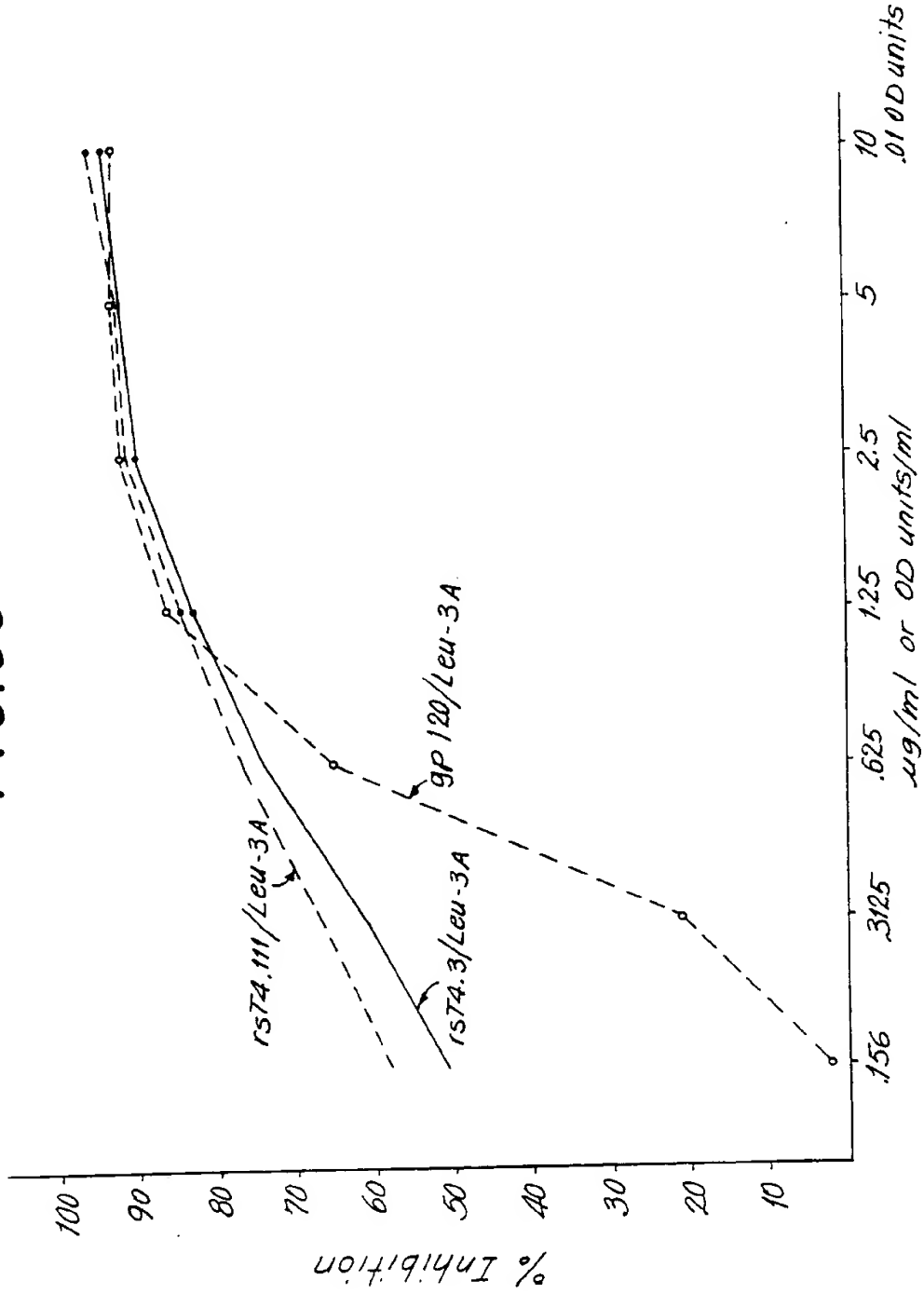

% Inhibition
100
90
80
70
60
50
40
30
20
10
156
312.5
.625
1.25
2.5
5
10
.01 OD units
ug/ml or OD units/ml
gp 120/Leu-3A
rsT4.111/Leu-3A
rsT4.3/Leu-3A

FIG. 37

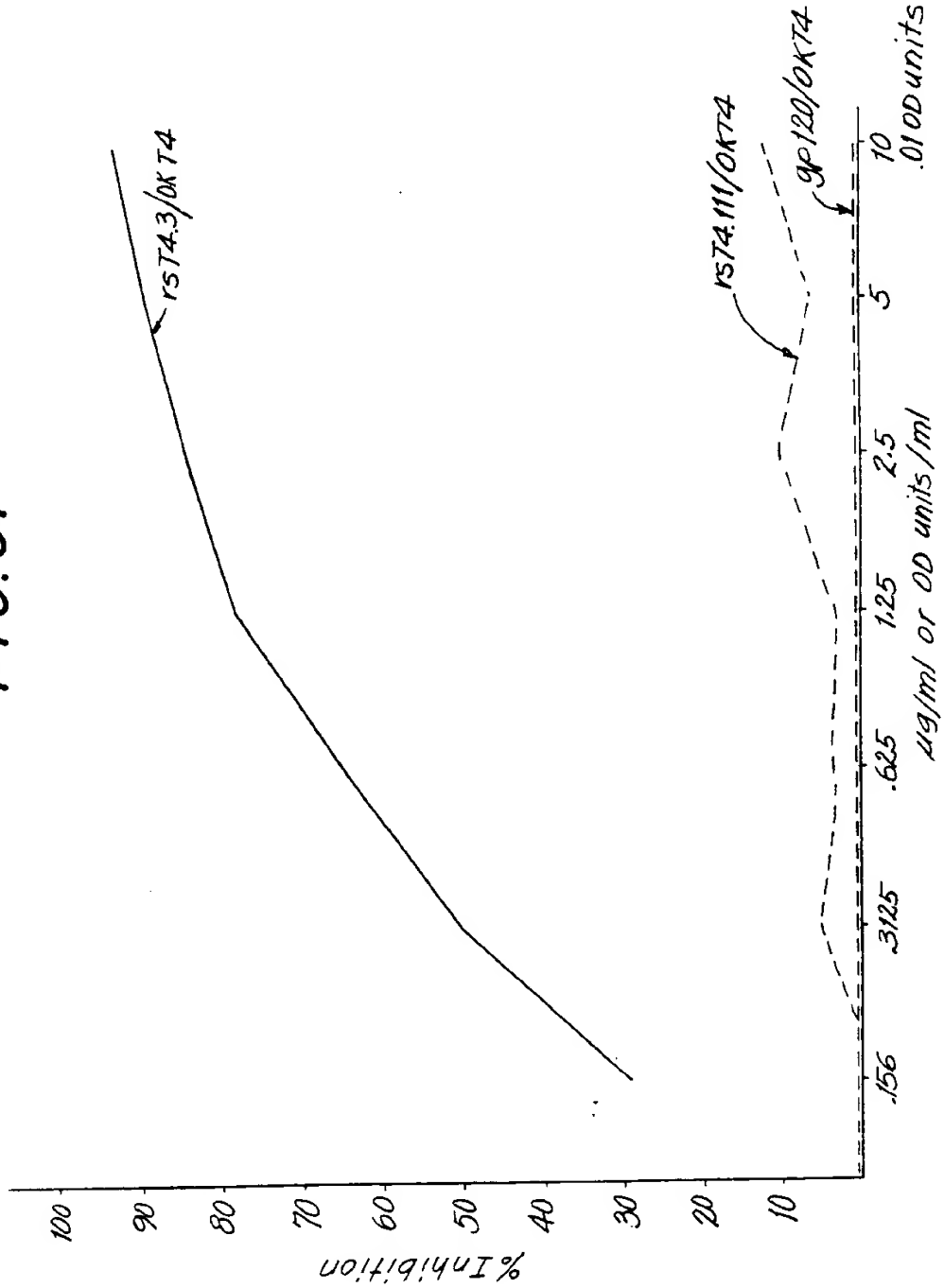

rsT4.3/0KT4
rsT4.111/0KT4
gp120/0KT4
% Inhibition
100
90
80
70
60
50
40
30
20
10
.156
.3125
.625
1.25
2.5
5
10
.01 OD units
μg/ml or OD units/ml

FIG. 38

Competition ELISA for rST4.113

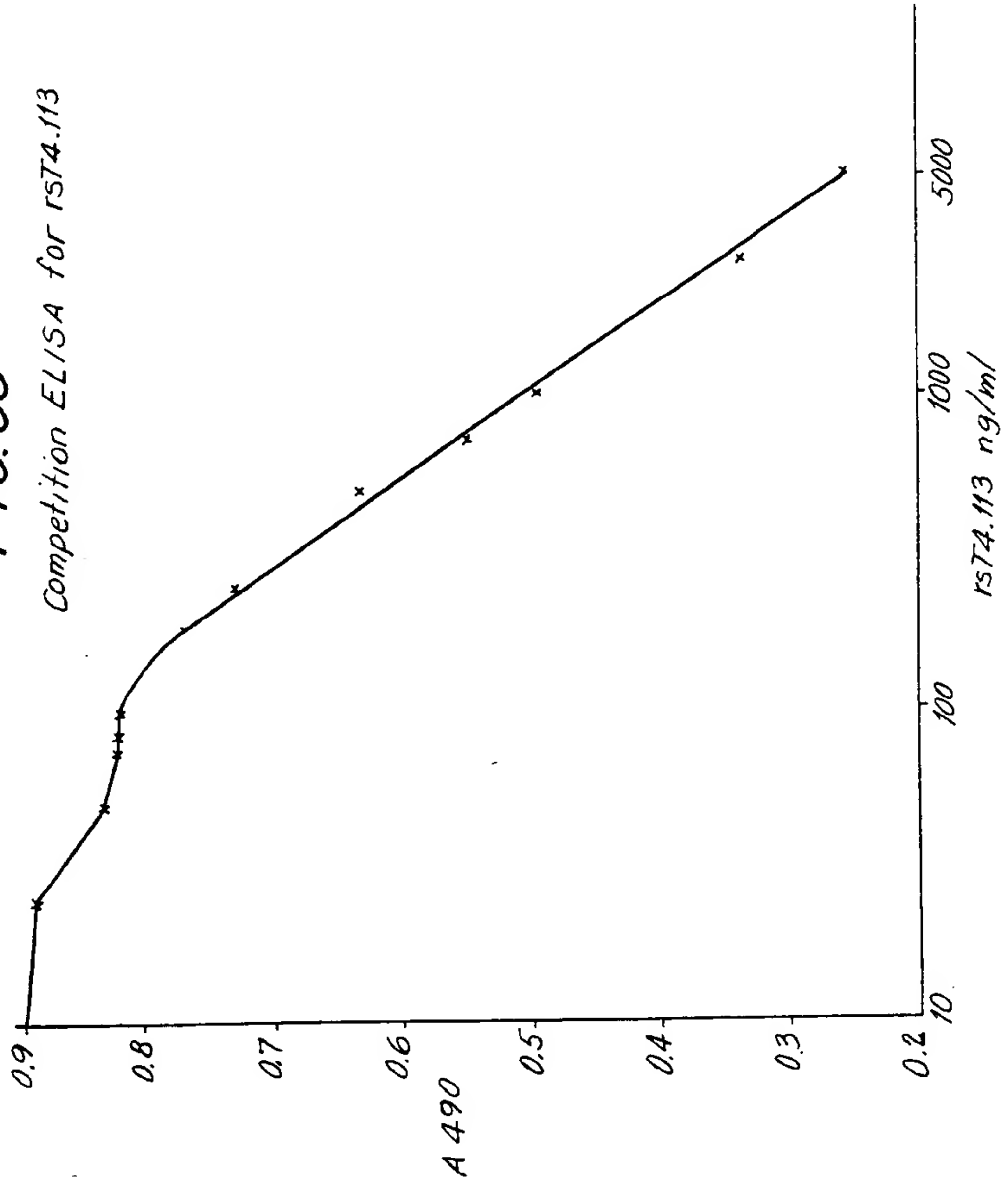


FIG. 39

P24 ANTIGEN RIA

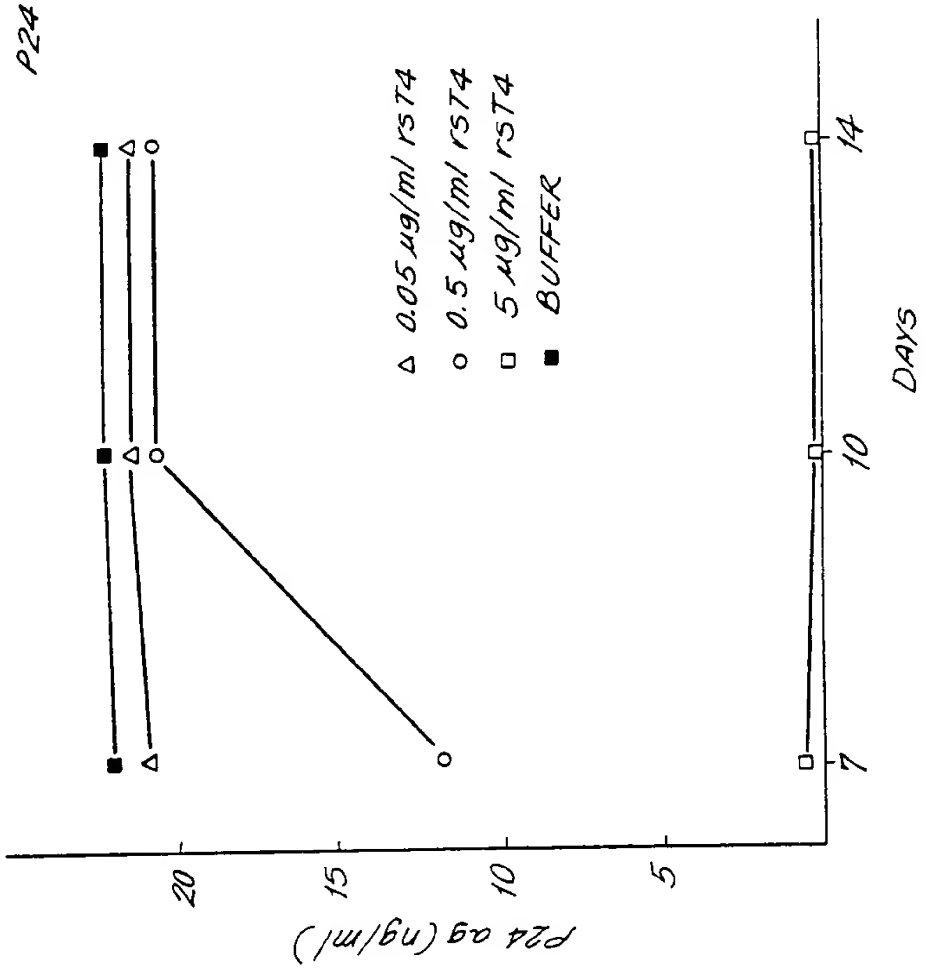


FIG. 40

C8166 CELL FUSION ASSAY

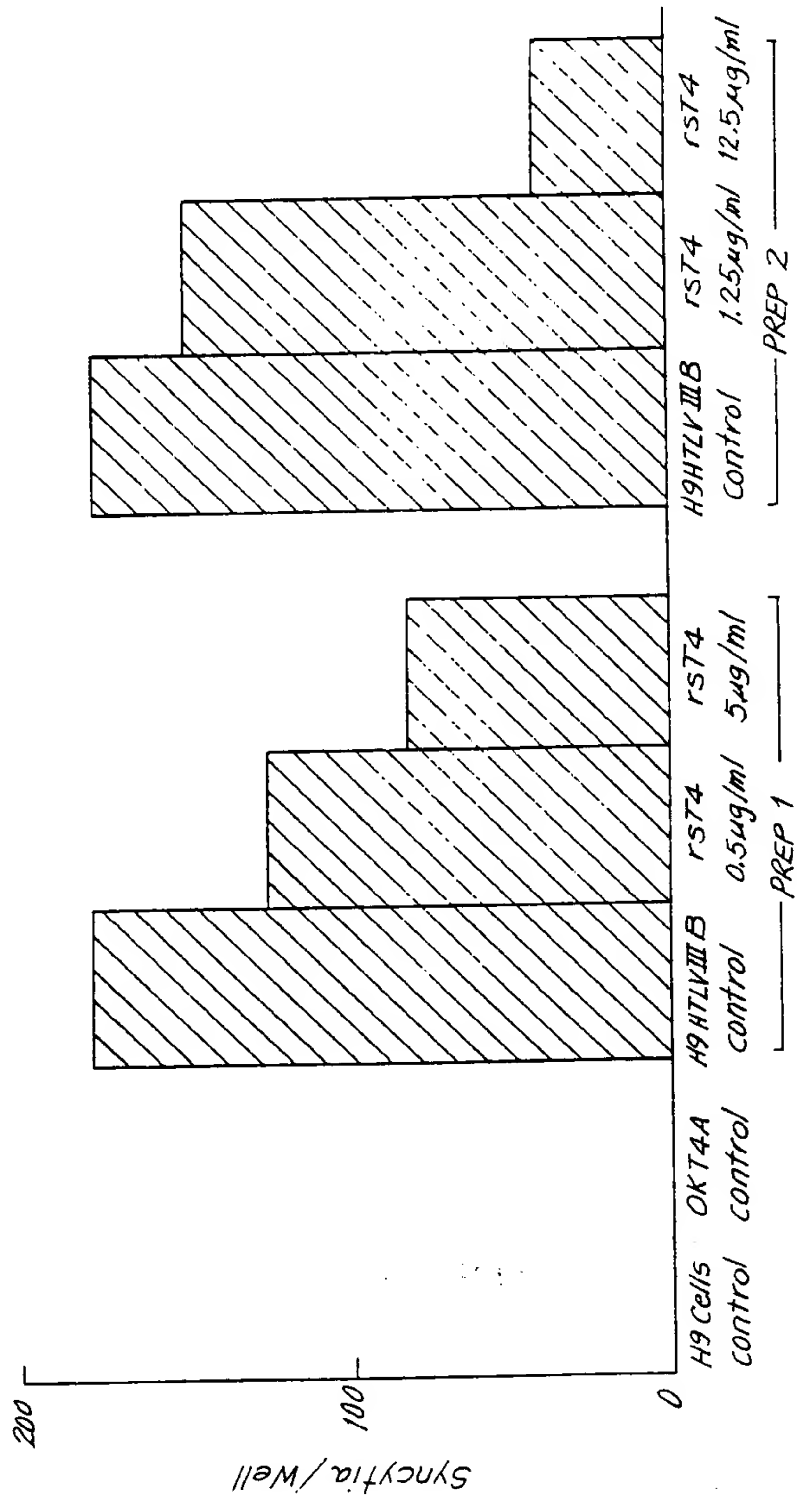


FIG. 41

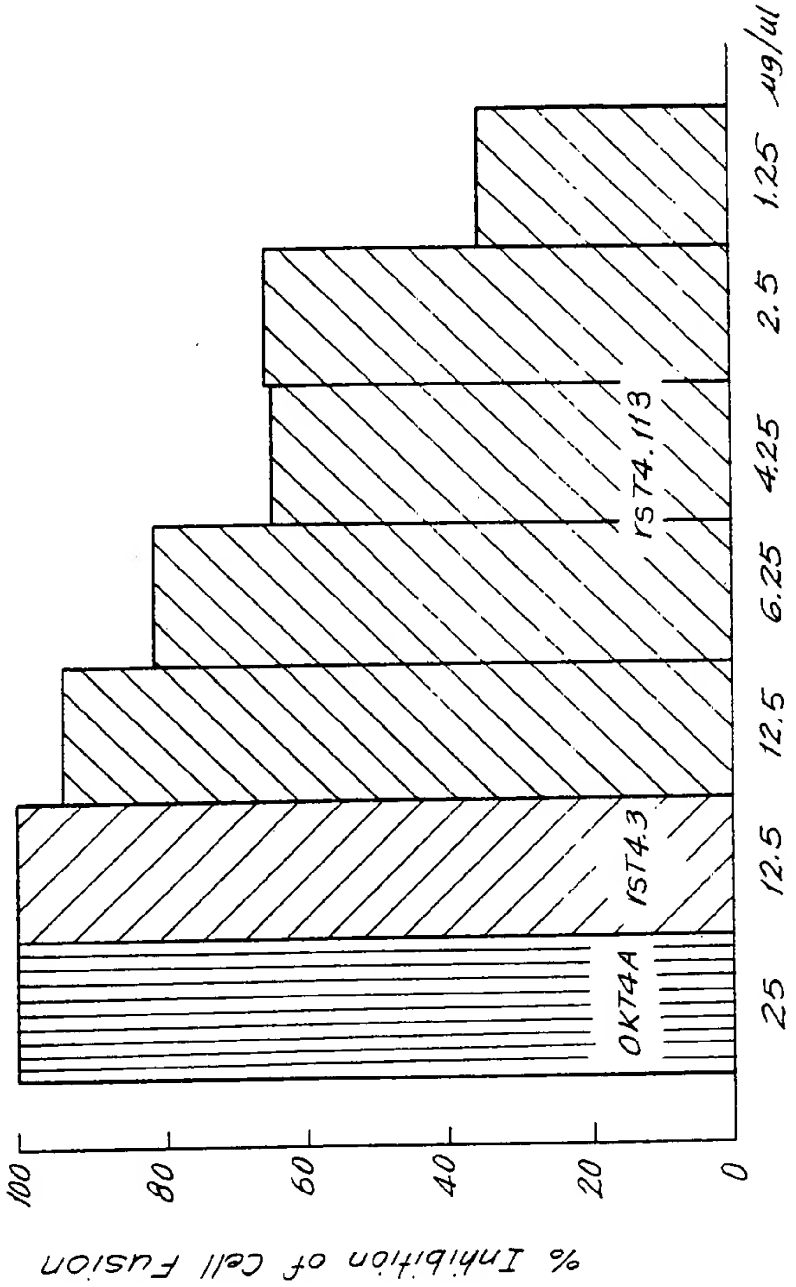
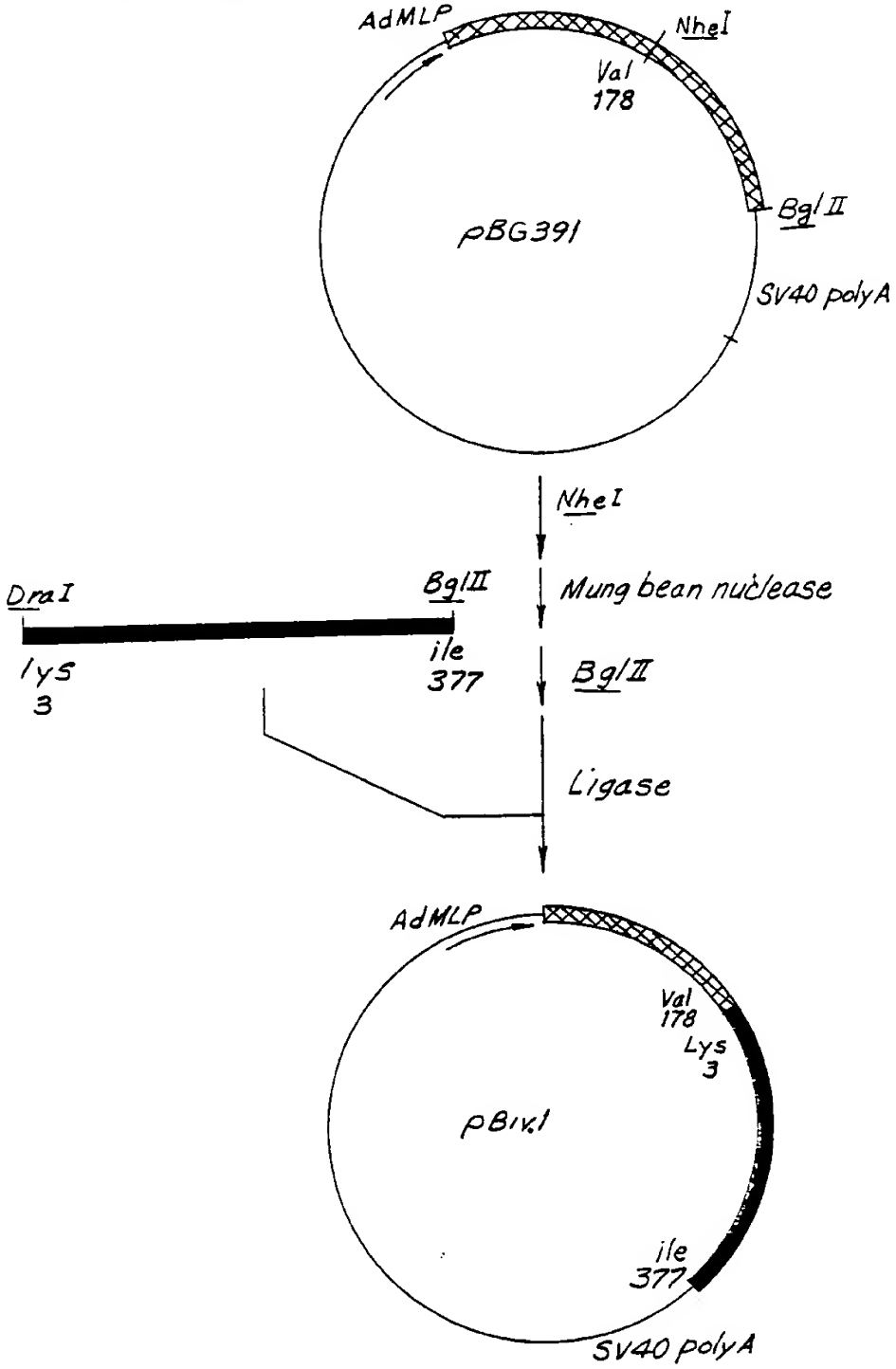

% Inhibition of cell Fusion
100
80
60
40
20
0
OKT4A
IST4.3
IST4.113
25
12.5
12.5
6.25
4.25
2.5
1.25
μg/μl

FIG. 42


AdMLP
NheI
Val
178
BglII
pBG391
SV40 polyA
NheI
Mung bean nuclease
BglII
Ligase
DraI
lys
3
BglII
ile
377
AdMLP
Val
178
Lys
3
pBIV.1
ile
377
SV40 polyA

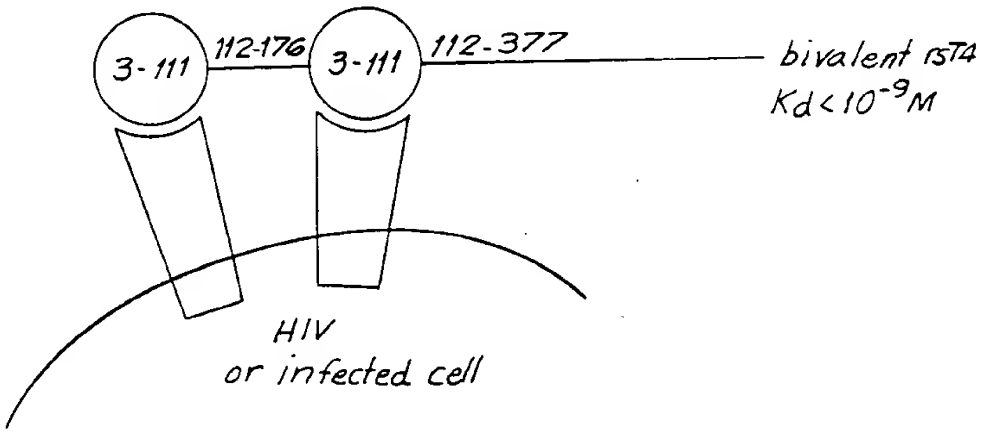

3-111
112-176
3-111
112-377
bivalent rsT4
Kd<10^-9 M
HIV
or infected cell

FIG. 43

International Application No: PCT/ US88 02940

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 95, lines 29-35 of the description
96, lines 6-11
96, lines 19-21

A. IDENTIFICATION OF DEPOSIT
Further deposits are identified on an additional sheet ☒ -- 3 additional sheets attached

Name of depositary institution
In Vitro International, Inc.

Address of depositary institution (including postal code and country)
611 (P) Hammonds
Ferry Road, Linthicum, Maryland 21090
United States of America

Date of deposit	Accession Number
See attached additional sheets	See attached additional sheets

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European patent is sought samples of the deposited microorganisms will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC).

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau

was 13 JANUARY 1989
(13.01.89)

J.-L. Barm
(Authorized Officer)

Form PCT/RO/134 (January 1981)

(January 1985)

Additional Sheet 1 of 3 To Form
PCT/RO/134

Continuation Of Box A

IDENTIFICATION OF DEPOSITS

BG378: E.coli MC1061/pBG378
199-7: E.coli MC1061/p199-7
170-2: E.coli JA221/p170-2
EC100: E.coli JM83/pEC100
BG377: E.coli MC1061/pBG377
BG380: E.coli MC1061/pBG380
BG381: E.coli MC1061/pBG381

DATE OF DEPOSITS

2 September 1987

ACCESSION NUMBERS

IVI 10143
IVI 10144
IVI 10145
IVI 10146
IVI 10147
IVI 10148
IVI 10149

Additional Sheet 2 of 3 To Form
PCT/RO/134

Continuation Of Box A

IDENTIFICATION OF DEPOSITS

BG-391:	E.coli MC1061/pBG391
BG-392:	E.coli MC1061/pBG392
BG-393:	E.coli MC1061/pBG393
BG-394:	E.coli MC1061/pBG394
BG-396:	E.coli MC1061/pBG396
203-5:	E.coli SG936/p203-5

DATE OF DEPOSITS

6 January 1988

ACCESSION NUMBERS

IVI 10151
IVI 10152
IVI 10153
IVI 10154
IVI 10155
IVI 10156

Additional Sheet 3 of 3 To Form
PCT/RO/134

Continuation Of Box A

IDENTIFICATION OF DEPOSITS

211-11: E.coli A89/pBG211-11
214-10: E.coli A89/pBG214-10
215-7: E.coli A89/pBG215-7

DATE OF DEPOSITS

24 August 1988

ACCESSION NUMBERS

IVI 10183
IVI 10184
IVI 10185

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/02940

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): C07H 15/12; C12Q 1/70, C12Q 1/02, see attachment.
U.S. CL.: 536/27; 435/5,29,39,68,91,170,172.3, see attachment.

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	435/5,29,39,68,91,170,172.1,172.3,240,253,320; 530/350,412; 514/2; 424/85; 536/27; 935/6, 9, 11,12,15,22,23,24,59,60,65,66

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

Chemical Abstract Data Base (CAS) 1967-1988; Biosis Data Base 1969-1988 Keywords: CD4, T4, TCell, AIDS, HTLV, HTLVI,HTLVIII, ~~see attachment.~~

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	SCIENCE, Volume 234, issued 1986 November, (Washington, DC., U.S.A.), (Q.J. SATTENTAU ET AL), "Epitopes of the CD4 Antigen and HIV Infection" See pages 1120-1123. See particularly page 1120	13-20, 29-33 and 48-52
Y	SCIENCE, Volume 234, issued 1986, November, (Washington, D.C. U.S.A) (J.A. HOXIE ET AL), "Alterations in T4 (CD4) Protein and mRNA synthesis in Cells Infected with HIV" see pages 1123-1127. See particularly page 1123.	13-20, 29-33, and 48-52
Y,P	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A, Volume 84, issued 1987 December (Washington, D.C., U.S.A), (P.J. MADDON ET AL.), "Structure and Expression of the Human and Mouse T4 Genes", See pages 9155-9159, See particularly page 9155 and 9156.	1-4,25-27, 34-36 and 39-46

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

28 NOVEMBER 1988

Date of Mailing of this International Search Report

03 FEB 1989

International Searching Authority

ISA/US

Signature of Authorized Officer

Richard C. Peet

RICHARD C. PEET

Form PCT/ISA/210 (second sheet) (Rev.11-87)

Attachment to PCT/ISA/210
I. Classification of Subject Matter

IPC: C12Q 1/06, C12P 21/00, C12P 19/34, C12P 1/04, C12N 15/00, C12N 7/00; C07K 13/00, C07K 3/00; A61K 37/68; A61K 39/00, A61K 45/02

US.CL.: 240, 320; 530/350, 412; 514/2; 424/85

II. Fields Searched

Keywords: ARC, Surface, receptor, therap?, purif?, Immunoassay, Detection, Pharmaceutical Composition, Lymphocyte, Igg, Polyvalent, Solub?, gene, Clon?, Protein, Polypeptide, Fusion, Expression, Vector, Plasmid, Surface Protein, Surface Antigen, Acquired Immune Deficiency Syndrome, Retrovirus

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	PROCEEDINGS NATIONAL ACADEMY OF SCIENCES, U.S.A, Volume 84, issued 1987, June (Washington, D.C., U.S.A.), (T.C. CHANH ET AL.), "Monoclonal Anti-idiotypic Antibody Mimics the CD4 Receptor and Binds Human Immunodeficiency Virus" See pages 3891-3895. See particularly page 3891.	13-24, 29-33 and 48-52
X Y	CELL, Volume 47, issued 1986, November, (Cambridge, Mass., U.S.A) (P.J. MADDON ET AL), "The T4 Gene Encodes the AIDS Virus Receptor and is Expressed in the Immune System and the Brain", See pages 333-348, See particularly pages 333-335.	1,3-6 and 25-27 2,7-24 and 28-50
P,Y	CHEMICAL ABSTRACTS, Volume 107, no. 15, issued 1987 October 12 (Columbus, Ohio, U.S.A), T.L. LENTZ et al, "Rabies virus binding to cellular membranes measured by enzyme immunoassay' see page 359, column 1, the abstract no. 131853f, Muscle Nerve, 1985, 8(4), 336-345 (Eng).	16-18 32-33 and 50
Y	CHEMICAL ABSTRACTS, Volume 106, no. 21, issued 1987, May 25, (Columbus, Ohio, U.S.A), J.P. ZIMMER ET AL., 'Diphenylhydantoin (DPH) blocks HIV-receptor on T-lymphocyte surface', see page 123, column 1, the abstract no. 168522c, Blut, 1986, 53(6), 447-450 (Eng).	13-15, 19-20, 29-30, 48-49 and 51-52
Y,P	BIOLOGICAL ABSTRACTS, Volume 85, no. 4, issued 1988, April 15 (Philadelphia, PA, U.S.A), A.G. DALGLEISH ET AL., 'Neutralization of HIV isolates by anti-idiotypic antibodies which mimic the T4 (CD4) epitope: A potential AIDS vaccine' see page 222, abstract no. 37595, Lancet 2 (8567): 1047-1050 (Eng).	13-15, 19-20, 29-30, 48-49, and 51-52